EXPERIMENTAL CELL RESEARCH

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CROSS REACTION OF SEROTYPES 51 A, 51 B, AND 51 D OF *PARAMECIUM AURELIA*, VARIETY 4[1]

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The study of the serotypes of *P. aurelia* has thus far been restricted to investigations of those antigens which are supposedly associated with the cilia, since visual observation of the immobilization reaction has been the only criterion used. Important conclusions have been drawn from these studies (5), but these conclusions are not *a priori* applicable to antigen systems not associated with the structures on the periphery of the cell. It was therefore of considerable interest to investigate other possible antigen systems in *P. aurelia*. The investigations were limited to three serotypes, 51 A, 51 B, and 51 D. These three serotypes all belong to a single strain of *P. aurelia*, variety 4 (strain No. 51), i.e. they are descended from a single individual collected in nature. The origin and nature of the diverse serotypes have been fully described. Since we were fundamentally interested in the antigenic mosaic of the different serotypes the serological reactions of the living animals were compared with homogenates. The quantitative adsorption technique was the method of choice.

MATERIAL AND METHODS

Only the general techniques used in this investigation will be described here. Special determinations will be given in the results section. Methods for growing serologically pure cultures of *P. aurelia*, the preparation of antisera and the titration of these antisera have already been described (4). *Paramecium aurelia* var. 4, stock 51.7 (killers) of serotypes A, B and D were grown in mass cultures. The cultures were checked for purity with regard to antigen type by titrating samples of approximately 10 000 animals with the appropriate sera. A one per cent impurity was tolerated. Preliminary concentration of the cultures was achieved by filtration through a Berkefeld candle till the volume was reduced from 20 l to approximately 750 ml. At this stage the usual concentration of animals was approximately 25 000 per ml. The animals were then washed through exhausted sterile culture fluid and

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concentrated by electromigration in the apparatus described in (6). The final suspension of *P. aurelia* (10–15 ml) was frozen in a dry ice-acetone mixture and lyophylized. The dried preparations were stored at —12° until needed.

For the quantitative adsorption tests small amounts of the dried preparations were suspended in Ringer's solution, diluted 1:10 and homogenized in a hand-operated aluminum colloid mill or with a micro macerator (2). Several dilutions of this homogenate were made up. For the test 1 ml of homogenate was mixed with 1 ml of serum diluted 1:2. The mixture was incubated at 32° for 40 minutes with stirring by convection. The tubes were then left for 24 hours at 0°, and were centrifuged for one hour at 0° at 24 000 $\times$ g in an angle centrifuge. The supernatant was carefully poured off and the tubes were allowed to drain for 10 minutes. The precipitate was stirred up in 2 ml of ice-cold Ringer's solution (1:10) and recentrifuged for one hour. The process of draining and centrifuging was repeated once more. The nitrogen of the sediments was determined with the micro Kjeldahl technique of Pregl (3), using the digestion mixture of Brüel *et al.* (1). Each series of determinations included the following sets in duplicate: 1. one ml of homogenate; 2. one ml of homogenate in control serum; 3. one ml of homogenate in each antiserum.

OBSERVATIONS

The following data were derived from these determinations: 1. N content of the homogenate; 2. N content of the sediment in control serum (a); 3. N content of the sediment in the antiserum (b); 4. N precipitated by the antigens ($b-a$); 5. the ratio $N_{\text{added}}/N_{\text{adsorbed}}\left(\frac{b-a}{a}\right)$. The ratio $N_{\text{added}}/N_{\text{adsorbed}}$ is an arbitrary figure. It can be seen from Tables II–IV that the N content of the sediment in control serum is lower than the N content of the corresponding homogenate. The supernatant of the homogenate treated with control serum contains undoubtedly soluble N. Part of this is antigenic and is precipitated and determined in the sediment obtained by treating the homogenate with antiserum. This antigenic N escapes detection in the control serum-treated homogenates. Since the amount of soluble, non sedimentable N is approximately the same for all serotypes, the N value obtained from control serum-treated homogenates was used as a measure of the antigen N. It is fully realized that this value does not represent the total antigen N present in the paramecia.

RESULTS

The characteristics of the antisera used in these investigations are given in Table I. The adsorptions of 51 A, 51 B and 51 D serotypes in 51 A, 51 B and 51 D antisera are given in Tables II–IV. The difference between

TABLE I

Characteristics of the 51 A, 51 B and 51 D Sera.

	Serum 51 A			Serum 51 B			Serum 51 D		
Dilution	51 A	51 B	51 D	51 A	51 B	51 D	51 A	51 B	51 D
1 : 50	+	ret	–	–	+	–	–	–	+
1 : 100	+	ret	–	–	+	–	–	–	+
1 : 200	+	–	–	–	+	–	–	–	+
1 : 400	+	–	–	–	+	–	–	–	+
1 : 800	+	–	–	–	+	–	–	–	+
1 : 1 600	+	–	–	–	Slug	–	–	–	Slug
1 : 3 200	Slug	–	–	–	Slo	–	–	–	V. Slo
1 : 6 400	Slo	–	–	–	ret	–	–	–	Slo
1 : 12 800	ret	–	–	–	ret	–	–	–	ret

In this table and in table V the abbreviations stand for: — not affected; ret = retarded; Slo = Slow; V. Slo = Very Slow; Slug = Sluggish; + = immobilized.

TABLE II

Adsorption pattern of homogenates of serotype 51 A in 51 A, 51 B and 51 D antisera.

	N 51 A sediment in control serum *a*	*Paramecium N* soluble in control serum Per cent	51 A antiserum			51 B antiserum			51 D antiserum		
			N sediment antiserum *b*	*N* adsorbed $b-a$	$\frac{b-a}{a}$	*N* sediment antiserum *b*	*N* adsorbed $b-a$	$\frac{b-a}{a}$	*N* sediment antiserum *b*	*N* adsorbed $b-a$	$\frac{b-a}{a}$
2	40.0	56.2	101.0	61.0	1.52	61.0	21.0	0.53	57.5	17.5	0.44
4	49.7	58.9	109.6	59.9	1.20	81.9	32.2	0.65	86.8	37.1	0.75
2	67.9	58.4	179.2	111.3	1.64	116.9	49.0	0.72	126.0	58.1	0.86
4	75.2	62.5	183.7	108.5	1.45	117.8	42.6	0.57	113.6	38.4	0.51
8	81.2	58.9	187.6	106.4	1.30	123.2	42.0	0.52	122.0	40.8	0.50
8	138.6	55.9	313.6	175.0	1.26	212.3	73.7	0.49	203.9	65.3	0.44
6	142.9	57.0	306.9	164.0	1.15	224.4	81.5	0.57	209.9	67.0	0.47
6	149.6	57.6	273.7	124.1	0.84	223.3	73.7	0.49	214.9	65.3	0.44

the titration data obtained with live animals and the adsorption data obtained with homogenates of lyophylized animals is great. In the case of the living animals, except for the slight cross reaction of 51 B animals with 51 A serum, no cross reactions are noted (Table I). However, when homogenates of lyophylized animals are used and the adsorption of antibody is measured, every type cross reacts to a great extent with the other sera,

TABLE III

Adsorption pattern of homogenates of serotype 51 B in 51 A, 51 B, and 51 D antisera.

N 51 B homogenate	*N* 51 B sediment in control serum *a*	*Paramecium N* soluble in control serum Per cent	51 A antiserum			51 B antiserum			51 D antiserum	
			N sediment antiserum *b*	*N* adsorbed $b-a$	$\frac{b-a}{a}$	*N* sediment antiserum *b*	*N* adsorbed $b-a$	$\frac{b-a}{a}$	*N* sediment antiserum *b*	*N* adsorbed $b-a$
67.2	39.2	58.3	84.0	44.8	1.14	88.9	49.7	1.26	87.0	37.8
87.7	50.4	57.3	101.3	50.9	1.00	115.5	65.1	1.27	89.5	39.1
130.2	79.1	60.8	158.2	79.1	1.00	153.3	74.2	0.94	134.4	55.3
162.4	90.3	55.6	171.5	81.2	0.90	177.7	87.4	0.97	162.4	72.1
162.1	100.2	61.8	202.7	101.5	1.01	211.9	111.7	1.10	176.6	76.4
212.3	120.4	56.7	235.8	115.4	0.96	247.5	127.1	1.15	203.4	83.0
250.6	146.9	58.6	282.4	135.5	0.93	274.3	127.4	0.87	239.3	92.4
262.5	151.2	57.6	293.3	142.1	0.94	313.9	162.7	1.07	287.1	135.9

TABLE IV

Adsorption pattern of homogenates of serotype 51 D in 51 A, 51 B and 51 D antisera.

N 51 D homogenate	*N* 51 D sediment in control serum *a*	*Paramecium N* soluble in control serum Per cent	51 A antiserum			51 B antiserum			51 D antiserum	
			N sediment antiserum *b*	*N* adsorbed $b-a$	$\frac{b-a}{a}$	*N* sediment antiserum *b*	*N* adsorbed $b-a$	$\frac{b-a}{a}$	*N* sediment antiserum *b*	*N* adsorbed $b-a$
55.9	34.2	61.2	39.9	5.7	0.17	60.2	26.0	0.76	64.4	30.2
89.2	53.1	59.5	57.1	4.0	0.07	69.1	16.0	0.30	101.4	48.3
124.2	68.2	54.9	74.6	6.4	0.09	105.0	36.8	0.54	112.7	44.5
129.6	70.0	54.0	77.0	7.0	0.10	103.4	33.4	0.48	136.5	66.5
133.9	80.4	60.0	83.0	2.6	0.03	130.8	50.4	0.58	177.8	97.4
173.2	102.0	58.9	111.3	9.3	0.09	153.3	51.3	0.50	173.3	71.6
199.3	120.4	60.4	132.5	12.1	0.10	183.4	63.0	0.53	231.4	111.0
246.4	135.5	55.0	141.4	5.9	0.04	176.5	41.0	0.31	277.3	141.8

with one exception, i.e. 51 D homogenates with 51 A serum. Type 51 A adsorbs considerable amounts of nitrogen from both 51 B and 51 D serum (Table II). The ratio $N_{\text{adsorbed}}/N_{\text{added}}$ in 51 B and 51 D serum is slightly less than half of the ratio in 51 A serum. Type 51 B adsorbs large amounts of antibody nitrogen from both 51 A and 51 B serum (Table III). The ratios $N_{\text{adsorbed}}/N_{\text{added}}$ are not significantly different in these

two sera. The same ratio is somewhat less in 51 D serum. The adsorption pattern of type 51 D on the three sera is different from the previous patterns (Table IV). This type adsorbs only very small amounts from 51 A serum and cross reacts to a great extent with 51 B serum.

The large difference between the titration data and the adsorption data could be interpreted so that the specificity of the antigens had been modified during the lyophylization process. Another interpretation could be that the homogenization of the animals had uncovered other antigens (somatic) which do not react with the immobilizing antibodies of the serum.

The evidence, that lyophylized and homogenized preparations of *P. aurelia* still contain antigens which can react with immobilizing antibodies is given in Table V. In these experiments the 51 A antiserum was treated with increasing amounts of homogenates, and the supernatant of the first centrifugation (containing the antiserum) was titrated with live 51 A animals in order to determine the decrease in titer of the serum. When 51 A serum was incubated with graded amounts of lyophylized and homogenized serotypes 51 A, 51 B, and 51 D, a reduction in titer of the 51 A serum of three dilutions resulted when 856.3 micrograms of the homologous type were used. A reduction of two dilutions of the titer of 51 A serum was found when the serum was incubated with the same amount of 51 B homogenate. However, no reduction was found when the serum was incubated with 51 D homogenate, although appreciable amounts of *N* were adsorbed from this serum. It must then be concluded that *P. aurelia* contains several antigens, those involved in the immobilization reaction, and those not involved. The latter might not be type specific.

The antigens present in homogenates of lyophylized *P. aurelia* can be separated into soluble and insoluble antigens. These two groups have different adsorption characteristics, as evidenced from the following series of experiments.

Lyophylized *P. aurelia* were homogenized in 2 ml of Ringer's solution (1 : 10). The homogenate was centrifuged in an angle centrifuge at 24 000 $\times$ g for 40 minutes. The supernatant was decanted carefully into a 10 ml volumetric flask. The sediment was resuspended in 2 ml of the dilute Ringer's solution, rehomogenized, and again centrifuged at 24 000 $\times$ g for 40 minutes. The supernatant was added to the previously obtained supernatant. The entire process was further repeated three times. All operations were carried out at 4° in the cold room. The volume of the combined supernatants was adjusted to 10 ml and this solution was recentrifuged if any flocculent material was present. The final sediment was rehomogenized and the volume was adjusted to 10 ml. Adsorption tests were carried out with the supernatant, the residual homogenate and the original homogenate.

TA

Adsorption of Antibody *N* and Reduction of Titer of Type 51 A Serum (1:4

N added γ	*N* adsorbed γ	51 A homogenate										*N* adsorbed γ			
		Serum Dilutions													
		12 ½	25	50	100	200	400	800	1 600	3 200	6 400		12.5	25	50
0	0	+	+	+	+	+	+	+	slug	slo	ret	0	+	+	+
40.0	61.0	+	+	+	+	+	+	slug	slo	ret	–	21	+	+	+
75.2	108.5	+	+	+	+	+	+	slug	slo	ret	–	42.6	+	+	+
142.9	164.0	+	+	+	+	+	+	slug	slo	ret	–	81.5	+	+	+
237.1	191.5	+	+	+	+	+	+	slug	slo	ret	–	114.1	+	+	+
328.3	217.6	+	+	+	+	+	slug	slug	slo	ret	–	160.3	+	+	+
445.1	294.7	+	+	+	+	+	slug	slug	slo	ret	–	228.1	+	+	+
494.2	301.4	+	+	+	+	+	slug	slug	slo	ret	–	245.1	+	+	+
659.1	291.5	+	+	+	+	+	slug	slug	slo	ret	–	240.1	+	+	+
856.3	281.4	+	+	+	+	slug	slug	slo	slo	–	–	205.1	+	+	+

The results are given in Table VI. Several facts are apparent from this table. First, homogenates of lyophylized paramecia contain large amounts of soluble nitrogen. Only part of this nitrogen is antigenically active. This is reflected in the low $N_{\text{adsorbed}}/N_{\text{no serum}}$ values. This soluble nitrogen is not sedimented at 24 000×g and therefore does not appear in the *N* value obtained for the sediment in control serum. Consequently, wherever this value was used for the calculation of the ratio of $N_{\text{adsorbed}}/N_{\text{no serum}}$ the ratios are higher than if the actual *N* content of the original homogenate had been used. It is evident, however, from the two ratio figures in the original homogenate (lines 4 and 5 of each block of Table VI) that the trend of cross adsorption remains the same.

Second, the soluble antigen fraction is more type specific than the insoluble fraction. The supernatant fraction of serotype 51 A cross reacts only to a slight extent with 51 D anti-serum, while the insoluble fraction cross reacts to a large extent with both 51 B and 51 D serum. The supernatant fraction of serotype 51 B cross reacts to some larger extent with both 51 A and 51 D sera. However, the insoluble fraction was non-specific. It cross reacts to a higher degree with either 51 A serum or 51 D serum, than with its homologous serum. The supernatant fraction of serotype 51 D did not cross react with 51 A serum, but adsorbed nitrogen from 51 B serum. The sediment was again less specific.

aded Amounts of lyophilyzed and homogenized Serotypes 51 A, 51 B and 51 D.

mogenate						*N* adsorbed γ	51 D homogenate									
lutions							Serum dilutions									
00	400	800	1 600	3 200	6 400		12 ½	25	50	100	200	400	800	1 600	3 200	6 400
+	+	+	slug	slo	ret	0	+	+	+	+	+	+	+	slug	slo	ret
+	+	+	slo	ret	ret	17.5	+	+	+	+	+	+	+	slug	slo	ret
+	+	+	slo	ret	ret	38.4	+	+	+	+	+	+	+	slug	slo	ret
+	+	+	slo	ret	–	67.0	+	+	+	+	+	+	+	slug	slo	ret
+	+	slug	slo	ret	–	87.4	+	+	+	+	+	+	+	slug	slo	ret
+	+	slug	slo	ret	–	89.6	+	+	+	+	+	+	+	slo	slo	ret
+	+	slug	slo	ret	–	107.8	+	+	+	+	+	+	+	slo	slo	ret
+	+	slug	slo	ret	–	115.4	+	+	+	+	+	+	+	slo	slo	ret
+	+	slug	slo	ret	–	125.5	+	+	+	+	+	+	+	slo	slo	ret
+	slug	slug	slo	ret	–	120.1	+	+	+	+	+	+	+	slug	slo	ret

DISCUSSION

It is evident from the above experiments that the antigenic make-up of the three serotypes under investigation is complex. This is not surprising, since many body constituents are antigenic. The usual method in preparing the antisera consists in injecting homogenates of the specific serotypes into a rabbit. The resulting serum must be complex serum, containing antibodies against the different antigens present in *P. aurelia*. No attempts have been made to identify the different antigens, although a separation into one soluble and one insoluble fraction has been obtained.

The adsorption of antibody from the serum is not specific by the *N*-adsorption test, in contrast to the specificity found when live animals are used for the titration of the sera. It is to be expected that the antigen fraction contained in the sediment is less type specific, since the sediment would contain many compounds and structures, such as nucleoproteins and nuclei which would be common to all types of this particular stock. However, even the sediment contains in certain cases (A and D) some type specific antigen which is reflected in the higher specific adsorption of the homologous serum.

The supernatant fraction contains, apart from such structures as cilia, large amounts of soluble nitrogen from the animal. Unquestionably, part

TABLE VI

Type and Preparation	N no serum γ	N control serum γ	51 A serum (1 : 800)		51 B serum (1 : 800)		51 D serum (1 : 800)	
			N adsorbed γ	$\frac{N_{ads}}{N_{no\ ser.}}$	N adsorbed γ	$\frac{N_{ads}}{N_{no\ ser.}}$	N adsorbed γ	$\frac{N_{ads}}{N_{no\ ser.}}$
51 A								
Supernatant	226.8	0	72.1	0.32	28.9	0.13	13.3	0.05
Sediment	109.2	115.5	81.3	0.75	46.2	0.43	52.5	0.49
Total	336.0		153.4	0.46	75.1	0.22	65.8	0.20
Original homogenate	324.4	149.6	124.1	0.39[1]	73.7	0.23[1]	65.3	0.20[1]
				0.84[2]		0.49[2]		0.44[2]
51 B								
Supernatant	55.0	0	30.2	0.55	41.2	0.75	5.6	0.12
Sediment	82.9	81.2	45.0	0.54	30.8	0.37	40.6	0.49
Total	137.9		75.2	0.55	72.0	0.53	46.2	0.34
Original homogenate	130.1	79.1	78.7	0.61[1]	74.3	0.57[1]	55.1	0.43[1]
				1.00[2]		0.94[2]		0.70[2]
51 D								
Supernatant	103.6	0	0	0	21.7	0.21	39.9	0.39
Sediment	134.4	112.7	36.4	0.32	29.4	0.23	51.8	0.41
Total	237.4		36.4	0.15	51.1	0.22	91.7	0.39
Original homogenate	246.9	135.5	5.9	0.02[1]	41.0	0.17[1]	141.8	0.57[1]
				0.04[2]		0.30[2]		1.05[2]

[1] $\frac{N \text{ after adsorption} - N \text{ control serum}}{N \text{ homogenate}}$

[2] $\frac{N \text{ after adsorption} - N \text{ control serum}}{N \text{ control serum}}$

of this soluble nitrogen is non-antigenic. The part that is antigenic is more type specific than the sediment. The supernatant from serotype 51 A cross reacts to a slight extent with 51 D serum, but to a larger extent with 51 B serum. Similarly the 51 D supernatant cross reacts with 51 B serum, but not at all with 51 A serum. The supernatant of serotype 51 B cross reacts to a greater extent with 51 A serum than with 51 D serum.

It was not possible in these exploratory experiments to distinguish between the various antigenic and non-antigenic nitrogenous compounds. For this reason the nitrogen content of the sediment in control serum was arbi-

trarily considered to represent the antigen nitrogen. It is clear from Table VI that this figure does not represent the actual antigen nitrogen. However, since the calculated values of the antigen/antibody ratios were only considered in order to represent the trend in the cross reactivity of the different serotypes, the use of the above mentioned ratio was justified. The experiments summarized in Table VI permitted another approximation of the antigen/antibody ratio. There is a close correlation between the calculated ratios (3rd line of each block) and the actual determined ratios (4th line).

SUMMARY

Homogenates of lyophylized preparations of serotypes 51 A, 51 B and 51 D of *Paramecium aurelia* cross react to a large extent with the sera prepared against each type. This is in contrast to the high specificity found when live animals are used for the titration. The homogenates can be separated into a fraction that is not sedimented at 24 000 × g and a fraction that is sedimented. The latter fraction is much less type specific than the soluble fraction.

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A CULTURE CHAMBER FOR THE CONTINUOUS BIOCHEMICAL AND MORPHOLOGICAL STUDY OF LIVING CELLS IN TISSUE CULTURE[1]

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The parallel study of the biochemistry and morphology of living cells has long been the ambition of both biochemists and morphologists. With the recent advent of phase contrast microscopy, coupled with the well developed techniques of tissue culture, this ambition is realized in principle. It is now possible to study living, unfixed cover slip cultures of many types of cells and to observe the intracellular organs of these cells with no question of fixation artefacts. With this combination of techniques it should now be possible to study the "pharmacology" of chemical substances on the basis of the type of cell and even the particular part of the cell which is influenced by the compound in question. Conversely, much can be learned of the causal biochemical reactions associated with various cellular processes and structures by administering compounds whose biochemical action is known.

However, one further device or technique is required for the complete realization of this biochemical study of cells in tissue culture. A method must be developed which meets the rather rigid requirements for optimum growth and cellular activity of the cultures. These requirements are primarily: (a) sterility and (b) optimum conditions of temperature, nutrition and oxygenation. The same method must also meet the optical requirements of phase contrast microscopy, which are: (a) The culture vessel must have optically flat and parallel surfaces. (b) These surfaces must be no more than 1 mm apart[2]. (c) The culture vessel and the culture must,

[1] This work was supported by a grant from the Damon Runyon Memorial Fund for Cancer Research.

[2] This requirement is based on the necessity of having the microscope condenser and objective at the critical distance from the cells for adequate phase contrast optics. The sum of these distances depends on the magnification and on the make of the microscope. For Bausch and Lomb oil immersion (97 ×) objective this distance is about 1.0 mm.

of course, be transparent. This includes the requirement that a continuous liquid phase fills the culture chamber during periods of observation. A final requirement of a biochemical-morphological technique is that biochemically interesting substances can be introduced and removed at will.

The use of the Gey plate (3) meets in part these requirements. The culture is mounted between two cover slips in a Gey plate and placed under the microscope. In this way it is possible to observe migrated cells with phase contrast optics, but only for relatively short times. The cellular activity is greatly modified under these circumstances, as is evidenced by the relative rarity with which mitosis is observed and by the failure of the cultures to grow or even to survive for more than a few hours. It is possible to introduce liquids between the cover slips, but once introduced they cannot be removed.

A more recent modification of the Gey plate (2), the formation of a perfusion chamber between two cover slips, goes further in meeting the requirements for an adequate biochemical-morphological method. The perfusion chamber is formed by paraffin side walls between two cover slips, one of which carries the culture explant. Inlet and outlet tubes pass through the paraffin walls so that solutions can be passed constantly through the culture chamber. The two major disadvantages of this device are its cumbersome yet fragile construction and the obvious difficulty of mounting the two cover slips parallel.

The present paper describes the construction and indicates the use of a culture chamber in which tissues can be grown for unlimited time and in which phase contrast observation is possible continuously throughout the culture period. The chamber also provides for the introduction and removal of any desired series of fluids. The device is easily and cheaply constructed and is simple and stable in operation; cultures are easily set up and show growth and cellular activity comparable to conventional hanging drop cultures; and finally, the device is permanent and is easily cleaned and sterilized.

CONSTRUCTION OF CULTURE CHAMBER

a. *Plastic Chamber.* The tissue culture slide shown in Fig. 1 is milled from 1/4 inch thick acrylic plastic sheet (first grade polished Plexiglass) to the dimensions shown in the working drawings of Fig. 2. The milling and cutting must be done slowly and under water cooling to prevent heating and its consequent permanent distortion of the thin plastic. The dimensions of Fig. 2 are critical for the use of

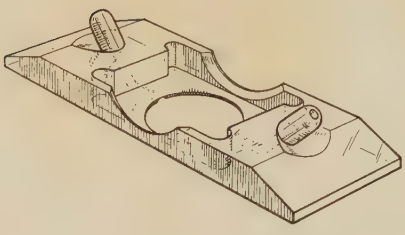

Fig. 1. Perspective drawing of plastic culture slide (without top and bottom cover slips).

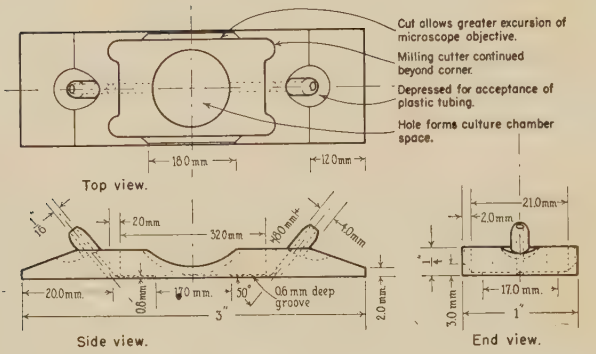

Fig. 2. Working drawings of plastic culture slide.

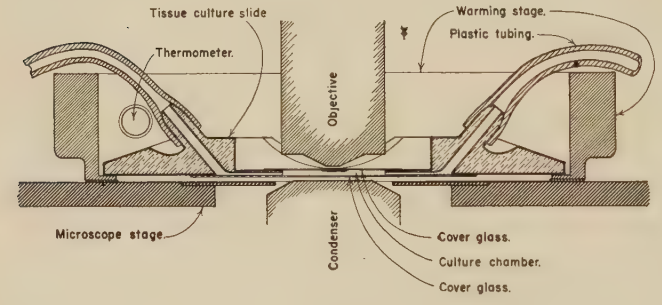

Fig. 3. Cross section of plastic culture slide with cover slips in place to form culture chamber. The microscope condenser, objective and stage and the incubator are indicated schematically.

the slide in a 1 inch by 3 inch stage incubator[1] and for maximum excursion of the objective over the culture chamber.

Two grooves are engraved in the under side of the block, extending from the circular opening to within 20 mm of each end of the block. At these points each groove joins a 1/16 inch hole drilled upward and outward through the block. Acrylic tubes are cemented on the upper surface so as to be continuous with these holes.

To complete the slide and form the floor and roof of the culture chamber, two No. 0 cover slips are attached with petrolatum or paraffin as seen in Fig. 3. The bottom cover glass must be wide enough to cover the 17 mm opening and at least

[1] Microscopic electrical incubator for oil immersion objectives, C. S. & E., A. S. Aloe Co., St. Louis.

50 mm long to enclose the grooves and holes cut in the plastic block. The top cover slip carries the culture and covers the entire circular opening.

b. *Glass Chamber.* For some purposes, contact of the perfusing fluid with plastic cannot be tolerated. To avoid this complication an all glass chamber with essentially the same principle of design was made. The chamber was made by grinding the holes and grooves in a 1 mm thick glass micro slide. This slide was then ground to a thickness of 0.8 mm. The inlet and outlet tubes were set in thicker glass blocks which were then attached to the slide by sodium silicate cement.

USE OF THE CULTURE CHAMBER

The glass chamber is sterilized by autoclaving; the plastic chamber by ultraviolet irradiation (1). The acrylic chamber cannot be sterilized by autoclaving nor in many germicidal solutions (including alcohol) because of damage to the plastic. Usual sterility measures for cover slips, solutions and culture explants are taken. The culture is started by placing the explant on the upper cover slip as in hanging drop cultures, then attaching the cover slip to the top of the slide with paraffin. A larger cover slip is attached with petrolatum to the bottom of the slide, converting the central opening to a culture chamber and the grooves to semicircular tubes (Fig. 3). The whole slide is then placed in a stage thermostat on the microscope. Nutrient fluid is introduced from a tuberculin syringe attached by Tygon tubing to the inlet tube. The outlet is connected to an empty syringe, giving control on both sides of the chamber so that no pressure or vacuum is created when the fluid is introduced or removed. To oxygenate the culture, the fluid is periodically replaced by air (comparable to the roller tube technique). When microscopic observations are made, the chamber must be filled with fluid. However, if desired, the fluid can be continuously flowing through the chamber without optical interference.

The effect of biochemical agents such as enzyme inhibitors, narcotics, and vital stains, is observed by replacing the simple nutrient fluid with a solution of the agent dissolved in the nutrient.

At the end of the observation the upper cover slip carrying the culture is removed and the culture fixed. The bottom cover slip is also removed, exposing all parts for easy and perfect cleaning.

OBSERVATIONS

Cultures have been grown continuously in this device and observed at convenient intervals for as long as three days. There is no experimental reason why they cannot be observed for much longer periods. Fig. 4 shows the activity of a single chick heart fibroblast photographed at 1 hour intervals from the 24th to the 32nd hour of culture. The changing shape, activity of the cell membrane, protruding and retracting of pseudopods, changes in shape and movements of the nucleus, and the activity of the nucleoli are apparent. The movements and shape changes of the cytoplasmic in-

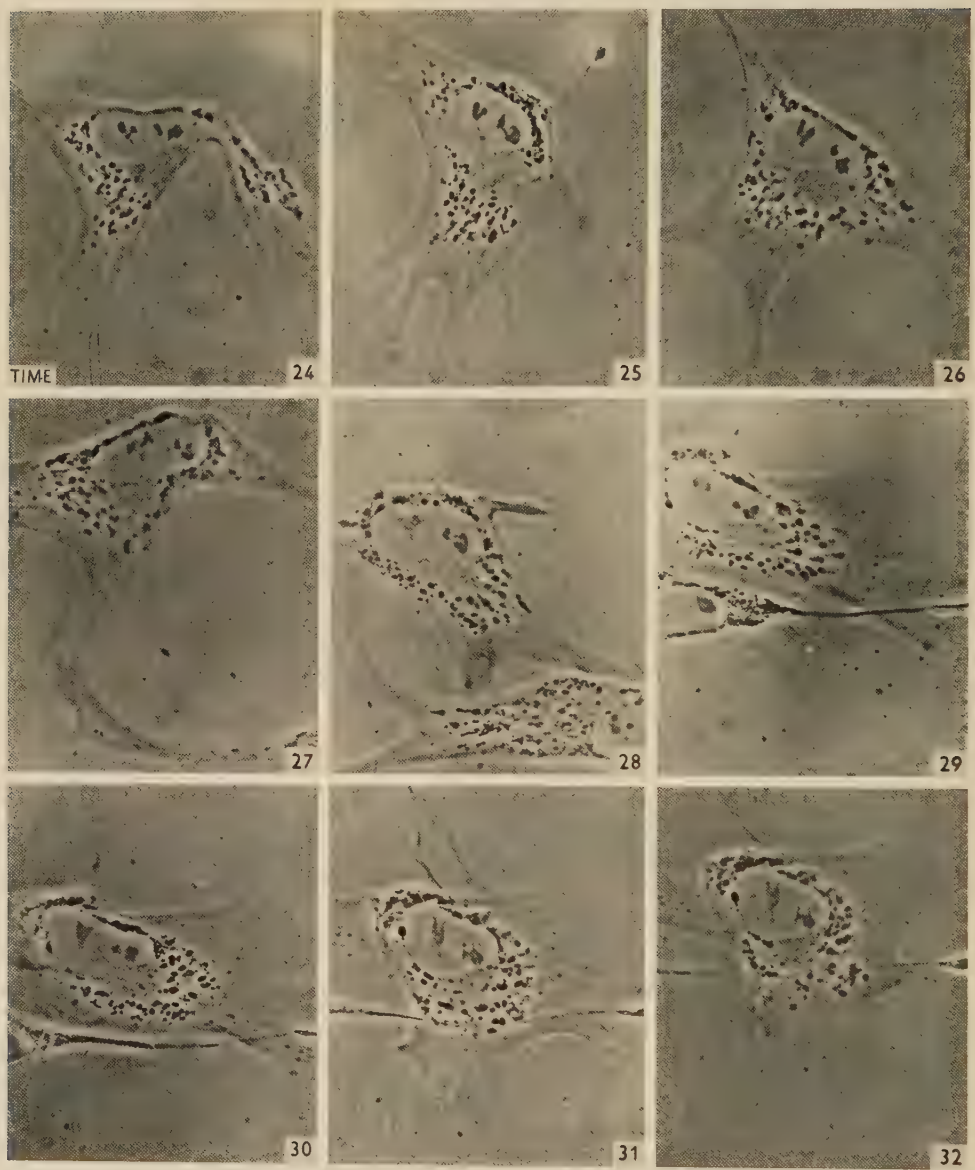

Fig. 4. Activity of a single chick heart fibroblast followed from the 24th to the 32nd hour of culture. Dark contrast phase photomicrographs, original magnification 1000 ×

clusions are not so obvious in this series of pictures. These more rapid cellular activities must be recorded by cinematographic methods, for which this culture chamber would be eminently satisfactory.

This chamber has been used to study the effect of cyanide and other enzyme inhibitors on these and other cellular activities. Vital stains have been introduced to cultures which have been examined first by phase contrast, then, after supravital staining, by ordinary bright field microscopy. In this way the intracellular organs as observed by phase contrast have been identified with their counterparts in stained cells. In a similar way specific enzyme reagents have been used to localize enzyme activities to particular intracellular organs. These observations will be published in detail elsewhere.

SUMMARY

A culture chamber has been described in which continuous phase contrast microscopic observation of growing tissue cultures can be made. The device meets the rigid requirements for optimum culture of tissues and for optical observation. The tissue can be exposed to biochemical reagents at will. Some of the observations made with this device have been described briefly.

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THE INTRACELLULAR DISTRIBUTION OF THE DEOXYPENTOSENUCLEASES OF MAMMALIAN TISSUES

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As part of an investigation of the enzymic mechanisms associated with the synthesis and metabolism of the nucleic acids, a study has been made of the distribution and properties of the deoxypentosenucleases[1] of certain mammalian tissues. The enzymic cleavage of the deoxyribonucleic acid molecules of the cell nucleus, as pointed out by Zamenhof and Chargaff (22), may be a phenomenon of considerable biological importance. Indeed, it has been suggested by Serra (21) that a system of (deoxyribonucleo)-depolymerases may be involved in the dispersion of the deoxyribonucleoprotein from the telophase chromosomes of dividing cells. The possibility that certain sulphydryl reactants and other mitotic poisons, which arrest mitosis in telophase, inhibit the action of an enzyme of the deoxyribonuclease type has also been discussed by Hughes (11).

The presence of deoxypentosenucleases in aqueous tissue extracts was established by Greenstein, Carter and Chalkley (9) and by Maver and Greco (16). In Maver and Greco's experiments it was found that these enzymes, which had optimal activity at pH 4.5, were activated by cysteine and were inhibited by the sulphydryl reactant sodium *p*-chloromercuribenzoate and by magnesium sulphate. The inhibitory action of magnesium ions (but not the activating effect of cysteine) on crude (i.e. unfractionated) preparations of thymus deoxypentosenuclease has also been reported by Brown, Jacobs and Laskowski (2).

Further evidence that the enzymes concerned with the intracellular metabolism of deoxyribonucleic acid differ from pancreas deoxyribonuclease was provided by the study of Zamenhof and Chargaff (22) on the deoxypentosenuclease of yeast and its specific cellular regulation. In this work it was demonstrated that 1.0 *M* sodium chloride extracts of crushed yeast contained a deoxypentosenuclease in combination with a specific inhibitor.

[1] This terminology, introduced by Zamenhof and Chargaff (22) is retained here in order to distinguish between the intracellular enzymes and the deoxyribonuclease of pancreas juice, the function of which is probably digestive.

On prolonged storage of the extracts in the cold the inhibitor was destroyed by the proteolytic enzymes of the yeast cell while the active deoxypentosenuclease was liberated into solution. The yeast deoxypentosenuclease, although heat-labile and requiring magnesium ions for activation, differed from pancreas deoxyribonuclease in so far as it was insoluble in water, but soluble in salt solutions, and possessed optimal activity at pH 6.0.

In the present paper experiments are recorded which confirm the presence of deoxypentosenucleases in homogenates of whole tissues. A study of the intracellular location of these enzymes indicates that they are confined to the cytoplasm of the cells. Methods for the isolation and purification of the deoxypentosenucleases, together with a summary of the properties of the purified preparations are recorded in the following communication.

EXPERIMENTAL

Tissues studied. The distribution of deoxypentosenuclease activity was studied in detail in calf thymus and mouse leukaemic tissue. The latter was a transplantable, acute lymphoblastic leukaemia in a hybrid FAK_1 strain, and was histologically uniform. The enzyme distribution was also determined in rat liver and mouse sarcoma (M 63) material.

Measurement of enzyme activity. Deoxypentosenuclease activity was determined viscosimetrically against a 0.2 per cent solution of a highly polymerized preparation of the deoxyribonucleic acid (N/P, 1.6) from calf thymus. Viscosity measurements were made in Ostwald viscometers (flow time for water (4.0 ml), 85–90 secs) at 34.5° ($\pm$0.25°). The substrate solution (2.0 ml), and the buffer (1.0 ml), or distilled water (1.0 ml) were mixed and brought to 34.5° in the viscometer. The enzyme solution (1.0 ml), preheated to 34.5° was then added, and the viscosity determined at frequent intervals.

Unit of enzyme activity. Deoxypentosenuclease activity was expressed in the units defined by McCarty (17). The use of the same preparation of thymus deoxyribonucleic acid for the activity determinations overcomes Little and Butler's (14) criticism of the McCarty unit of enzyme activity.

Deoxypentosenuclease activity of homogenates of whole tissue. Following the method briefly described by Zamenhof and Chargaff (22) the tissue was homogenized in a high-speed mixer with twice its weight of distilled water for 3–5 mins. After the addition of toluene (5 ml) the suspension was kept at 4° or 37°.

Toluene was chosen as a preservative in preference to sodium ethyl mercurithiosalicylate (merthiolate) (cf. 22), since the deoxypentosenuclease activities of such homogenates were consistently greater than those prepared in the presence of merthiolate. As the latter was without inhibitory action on the purified deoxypentosenucleases (cf. the following paper), it appears possible that toluene exerted a favourable influence on the liberation of the enzyme by concentrating inhibitory lipid-containing material at the hydrocarbon-water interface (cf. (19)).

Table I

Deoxypentosenuclease activities of homogenates of whole tissues.

Homogenate of	Deoxypentosenuclease activity (units/ml)
Calf thymus immediately after preparation	0.02
Calf thymus after 9 days at 4°	0.28
» » » 24 » » »	0.37
» » » 30 » » »	0.35
» » » 3 » » 37°	0.60
» » » 7 » » »	0.27
Mouse sarcoma (M 63) after 16 days at 4°	0.20
» 30 » » »	0.16
Mouse leukaemic tissue immediately after preparation	0.0
after 5 days at 4°	0.13
» 11 » » »	0.19
Rat liver after 5 days at 4°	0.32
» 11 » » »	0.36

At suitable intervals a fraction of the tissue suspension was centrifuged for 30 mins. at 4 500 r.p.m., the supernatant solution filtered through a plug of cotton-wool and an aliquot (1.0 ml) of the filtrate assayed for deoxypentosenuclease activity.

A freshly prepared homogenate was viscous and exhibited little or no deoxypentosenuclease activity. The viscosity of the suspension decreased on storage. The change in viscosity occurred slowly in homogenates kept at 4°; rapidly in homogenates incubated at 37°. Concomitantly with the decrease in viscosity, free deoxypentosenuclease activity appeared in the homogenates. Typical results (Table I) show that the enzyme activity of the tissue suspensions increased on storing to a maximum and then decreased. The fact that the deoxypentosenuclease activity of freshly prepared homogenates was low, together with the fact that the activity increased as the viscosity decreased suggested that the deoxyribonucleohistone of the tissue initially formed a substrate for the enzyme. This was confirmed experimentally by the finding that during the period of the decrease in viscosity, there occurred the degradation of deoxyribonucleic acid to dialysable, acid-soluble nucleotides.

Intracellular distribution of the deoxypentosenucleases.

Separation of nuclear and cytoplasmic fractions. To avoid possible artefacts through the redistribution of material the tissues were separated into nuclear and

cytoplasmic components by several methods. The "cytoplasmic fractions" remaining after the initial separation of the nuclei by centrifuging at low speed were recentrifuged for 5 mins. at 1 500 r.p.m. to remove any residual nuclei and then stored at 4° in the presence of toluene (5 ml). A suspension of the nuclei in water was then homogenized, if necessary, in the blendor, covered with toluene (5 ml) and kept at 4°. At suitable intervals, aliquots of both the nuclear and cytoplasmic fractions were centrifuged and the supernatant solutions assayed for deoxypentosenuclease activity.

1) *Fractionation of calf thymus.*

a) *in citric acid.* Thymus (100 g), freed from fat and connective tissue, was fractionated in dilute citric acid at *c.* pH 4.8 according to the method of Dounce (5). The cytoplasmic fraction obtained by this procedure was dialysed for 24 hours against three changes of distilled water (2 l) at 4° and then diluted with 0.2 *M* phosphate buffer pH 6.4 (1/5th vol.). The isolated nuclei were washed in water and suspended in 0.04 *M* phosphate buffer pH 6.4 (50 ml).

The deoxypentosenuclease activities (units/ml.×volume) of the nuclear and cytoplasmic fractions were as follows:—

	Total deoxypentosenuclease activity Time (days) at 4°			
	4	20	30	42
Cytoplasmic fraction.....	80	58	16	0
Nuclear fraction	0	0	0	0

b) *in 0.2 M phosphate buffer saturated with magnesium hydrogen phosphate at pH 6.4* (cf. 1, 12). The nuclear fraction, isolated from a homogenate of thymus (100 g) in the ice-cold solvent (500 ml), was purified by suspending in the solvent, straining through cloth and recentrifuging. This process was repeated several times. The final preparation of distorted nuclei, which was contaminated with some fibrous material (cf. (12)), in water (50 ml) formed a gelatinous suspension which was kept under toluene at 4°. No deoxypentosenuclease activity was detected in this fraction over a period of 25 days. In contrast, the cytoplasmic fraction contained a total of 270 units of deoxypentosenuclease activity after 2 days at 4°; after 10 days, 250 units and after 16 days, 210 units.

c) *in 0.88 M sucrose.* Thymus (100 g) was homogenized in 0.88 *M* sucrose (500 ml) and the homogenate fractionated as described by Hogeboom, Schneider and Pallade (10). The protein constituents of the cytoplasmic fraction, separated from the sucrose solution by precipitation with saturated ammonium sulphate solution (3 vols.), were dissolved in water (200 ml) and dialysed to remove ammonium sulphate. The residual solution was diluted with water to 400 ml and mixed with 0.2 *M* phosphate buffer, pH 6.4 (100 ml).

Deoxypentosenuclease activities, determined at intervals in aliquots of the nuclear and cytoplasmic fractions were as follows:—

	Total deoxypentosenuclease activity Time (days) at 4°		
	3	13	21
Cytoplasmic fraction	180	105	20
Nuclear fraction[1]	0	5	9

d) *in 0.88 M sucrose containing 0.0018 M calcium chloride.* The nuclei isolated from thymus (75 g) as described by Schneider and Peterman (20), were suspended in water (10 ml) and dialysed for 18 hours against running tap water. The resulting gelatinous suspension of swollen, distorted nuclei contained a weak deoxypentosenuclease activity (0.03 units/ml) after 8 days at 4°.

2) *Fractionation of mouse leukaemic tissue.*

With this material the cells were usually disintegrated by grinding with sand, rather than in the high-speed mixer. This method gave an efficient separation of the nuclei from the cytoplasm and was more convenient with the smaller amounts of tissue available for the experiments.

The deoxypentosenuclease activities associated with the separated cytoplasmic and nuclear fractions derived from the tumour tissue by fractionation in 0.14 *M* sodium chloride according to Claude's (3) method and by the above procedures a, b and c, are summarized in Table II.

3) *Fractionation of rat liver.*

Adult rat liver (40 g) was separated into the nuclear and cytoplasmic fractions by Claude's (3) method. The nuclei were suspended in water (30 ml) and 0.2 *M* phosphate buffer, pH 6.4 (5.0 ml) and kept at 4°. Assays for deoxypentosenuclease activity showed the nuclear fraction to contain a total of 4.8 units of activity after 24 hours, and 5.6 units after 6 days. The corresponding activities of the cytoplasmic fraction were 36 to 40 units respectively.

4) *Mouse sarcoma (M 63).*

Total units of deoxypentosenuclease activity of the nuclear and cytoplasmic fractions separated from the sarcoma tissue (15 g) by Claude's (3) method were 3.7 and 20.1 respectively after 16 days at 4°.

[1] In three further preparations of nuclei isolated from thymus (100 g) by this method the deoxypentosenuclease activities were, respectively, 0.09, 0.31 and 0.0 units/ml after 18 days at 4°.

TABLE II

Deoxypentosenuclease Activities of the Nuclear and Cytoplasmic fractions separated from Mouse Leukaemic Tissue.

Method of Fractionation	Fraction	Time (days) at 4°			
		3	10	17	28
		Total units of deoxypentosenuclease activity (expressed per 100 g tissue)			
Citric acid (Dounce, (5))	nuclei	0	–	0	–
	cytoplasm	–	35	–	17
0.2 *M* phosphate buffer saturated with $MgHPO_4$ at pH 6.4 (Brown *et al.*, (1))	nuclei	0	–	5	–
	cytoplasm	76	116	–	–
0.88 *M* sucrose (Hogeboom, *et al.* (10))	nuclei	0	7	–	12
	cytoplasm	–	91	–	–
0.14 *M* sodium chloride (Claude, (3))	nuclei	–	–	–	31
	cytoplasm	180	–	–	36

LOCATION OF THE DEOXYPENTOSENUCLEASES

The experimental results recorded in the previous section suggested that the deoxypentosenucleases found in homogenates of whole tissue were located in the cytoplasm of the cells. The fact could not be neglected, however, that low concentrations of the enzyme were associated with the nuclear fractions isolated by certain methods, since it seemed possible that any intranuclear deoxypentosenuclease would combine with its substrate during the isolation procedures. Under these conditions little or no free deoxypentosenuclease activity would be found in the nuclear fractions until the enzyme had degraded the cellular substrate.

It was not possible to determine the amount of deoxypentosenuclease activity associated with the nuclei by the difference between the total activity of a homogenate of the whole tissue and that of the cytoplasmic fraction isolated from the same weight of tissue, since the latter invariably contained the greater enzyme activity. Thus, in two independent experiments with calf-thymus, the cytoplasmic fraction isolated from the tissue (100 g) in 0.2 *M* phosphate buffer saturated with magnesium hydrogen phosphate at pH 6.4 (200 ml) contained 150 and 168 units of free deoxypentosenuclease activity after 7 days at 4°, whereas the homogenates of the whole

tissue (100 g) in the same solvent contained 64 and 114 units respectively after 7 days at 4°.

These results strongly suggest that in the preparation of a tissue homogenate, the cytoplasmic deoxypentosenuclease combines with its nuclear substrate.

It was shown above that the degradation of deoxyribonucleic acid to dialysable products occurred in tissue homogenates when these were kept at 4° or 37°. However, when isolated nuclei were kept for several weeks at 4°, little or no hydrolysis of the nucleic acid was observed. Thus, the aqueous suspension of thymus cell nuclei previously assayed for deoxypentosenuclease activity (sections b and c, p. 19) were kept for one month at 4°, centrifuged and the supernatant solutions dialysed for 48 hours against distilled water (800 ml). The dialysed solutions then were centrifuged to remove the precipitates formed on dialysis. Analysis of the clear supernatant solutions and of the dialysates, after concentration, with the Dische (4) diphenylamine reagent failed to show the presence of significant amounts of deoxyribose in these solutions. In contrast, aqueous suspensions of the precipitated material gave intensely positive reactions with the Dische reagent and also with the Sakaguchi reagent for arginine.

STABILITY OF ISOLATED NUCLEI

It was repeatedly observed throughout the work with suspensions of isolated nuclei that the latter were extremely unstable and readily disintegrated when transferred to distilled water. This was particularly noticeable with suspensions of nuclei isolated in 0.2 *M* phosphate or 0.88 *M* sucrose solutions. Presumably because of the precipitation of the nuclear deoxyribonucleohistone in the acid medium, nuclei isolated in citric acid according to Dounce's (5) method were more stable and maintained their form when suspended in distilled water provided the pH was maintained at about 5 with a few drops of 0.1 *M* citric acid. With increasing pH the nuclei first became swollen and gelatinous, and then agglutinated. With further increases in pH (i.e. above pH 6.0–6.3), microscopical examination revealed the presence of only a small percentage of shrunken nuclei (or nuclear residues) in a mass of amorphous material.

As an extension of the preceding study of deoxypentosenuclease distribution, it was of interest to determine whether the disintegration of isolated nuclei in aqueous suspension was an enzymic or physical process.

The effect of increase in pH on the stability of dilute suspensions of thy-

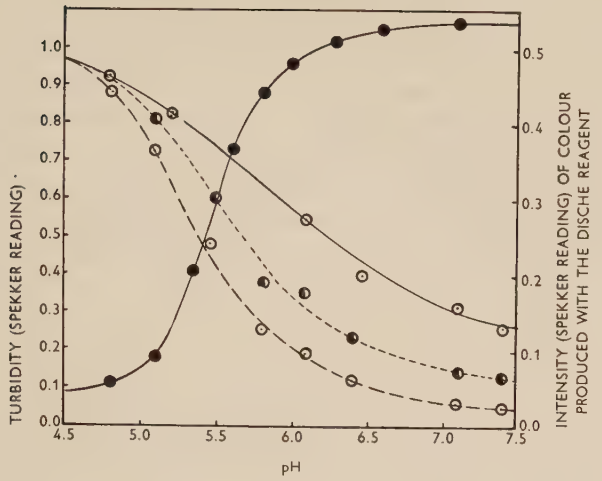

Fig. 1. The effect of increase in pH on the stability of dilute suspensions of thymus cell nuclei in *M*/15 (⊙———⊙) and *M*/75 (◐- - - - -◐) phosphate solutions. (○- - - - -○) Lysis in *M*/75 phosphate solutions after 1 hr. at 37°, and the liberation of soluble deoxyribonucleotides (●———●), as determined with the Dische (4) reagent.

mus cell nuclei isolated by the citric acid method is shown in Fig. 1. In these experiments, the nuclear suspension (0.1 ml $\equiv 1\times10^6$ nuclei) was added to a series of tubes containing distilled water (4.0 ml) and the appropriate *M*/15 phosphate buffer (1.0 ml). The pH values of the resulting suspensions were redetermined after the addition of the nuclei. The opacity of each suspension was measured in the Spekker Photoelectric Absorptiometer immediately after preparation of the series and also after incubation for 1 hour at 37°.

To determine whether the decreased opacity observed in these experiments was due to lysis of the nuclei and the dissolution of the nuclear contents, the liberation of soluble deoxyribonucleotides was followed by the Dische (4) reaction. The nuclear suspension (0.3 ml) was added to a series of tubes containing distilled water (4.0 ml) and *M*/15 phosphate buffer (1.0 ml) as described above. The suspensions were incubated for 1 hour at 37° and then centrifuged. Aliquots (2.0 ml) of the clear supernatant solutions were analysed for deoxypentose with the Dische reagent.

The results (Fig. 1) show that under the above conditions the dilute nuclear suspensions were stable below pH 5. Above this value clearing of the suspension occurred. This clearing increased rapidly with further increases in pH and was accompanied by a parallel rise in the deoxyribonucleo-

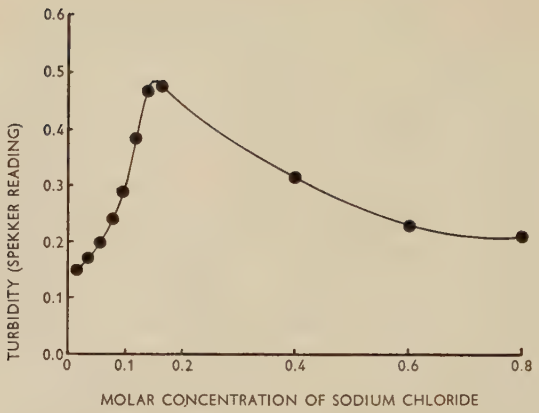

Fig. 2. Effect of sodium chloride on the lysis of dilute suspensions of thymus cell nuclei in *M*/75 phosphate buffer, pH 6.4.

tide content of the supernatant solution. Lysis immediately followed the addition of the nuclei to the buffer solutions and was little affected by incubation for 1 hour at 37°.

The lysis of the nuclei depended not only upon the pH, but also upon the ionic strength of the buffer solutions. Thus, the extent of the lysis of a nuclear suspension at a given pH was less in *M*/15 phosphate buffer than in a *M*/75 phosphate solution, while lysis at a given pH (i.e. 6.4) was markedly influenced by the addition of varying concentrations of sodium chloride (Fig. 2). The curve showing the variation of lysis with sodium chloride concentration (Fig. 2) exhibits maximum opacity (minimum lysis) at a sodium chloride concentration of 0.16 *M*, approximately parallels the well-known solubility curves obtained with isolated deoxyribonucleohistones in sodium chloride solutions (7, 18) and indicates that the dissolution of the nuclei is determined by the solubility of the deoxyribonucleohistone in the salt solutions.

The lysis of thymus cell nuclei (isolated with citric acid, 0.2 *M* phosphate buffer, or sucrose) in *M*/75 phosphate solutions of increasing pH was inhibited when the buffers contained a salt of a di- or trivalent metal (e.g. $CoCl_2$, $Ca(NO_3)_2$, $BeSO_4$, $La(OCOCH_3)_3$ $FeCl_3$ $Pb(NO_3)_2$, $AgNO_3$, $CuCl_2$, $MnSO_4$, $MgSO_4$) at a concentration of 0.002 to 0.0002 *M*. Lysis was also inhibited by solutions of iodine in potassium iodide (0.002–0.0001 *M*) and formaldehyde (0.05–0.1 *M*), but not by 0.002 *M* concentrations of such enzyme inhibitors as hydroxylamine, sodium citrate, sodium fluoride (cf. (6)), sodium arsenate, iodoacetic acid, sodium sulphide, potassium cyanide, cys-

teine and thioglycollic acid at pH 6.4, and sodium usnate at pH 8. No lysis occurred, however, when suspensions of nuclei, preheated at 60° for 15 mins. were adjusted to alkaline pH values.

The above results showed that the lysis of nuclei is prevented by those agents which denature or precipitate the nucleoprotein (e.g. heat, iodine, formaldehyde, and metallic ions (8)), but not by the range of enzyme inhibitors studied. It is concluded, therefore, that the lysis of isolated nuclei is a physical process, possibly resulting from osmotic forces due to the initial solution of the deoxyribonucleohistone.

DISCUSSION

The above observations, in agreement with those of Marshak (15), show that the breakdown of isolated (thymus) cell nuclei, in contrast to the breakdown of the whole tissue, is not accompanied by the degradation of the deoxyribonucleic acid to dialysable (poly)-nucleotides. They are also in accordance with the results of fractionation experiments which indicate that the deoxypentosenucleases found in homogenates of calf thymus, rat liver and mouse leukaemic and sarcoma tissue are confined to the cytoplasm of the cells. The enzyme activity which is associated with the nuclei isolated from tissues by certain methods (e.g. with sucrose solutions) is considered to be an artefact arising from the adsorption of the enzyme from the cytoplasm during the fractionation procedure. This follows from the following facts:

Deoxypentosenuclease activity is always associated with the cytoplasmic fractions, irrespective of the method of preparation, and, moreover, may be demonstrated in these fractions immediately after preparation. The deoxypentosenuclease activity of homogenates of whole tissues in distilled water (in which the nuclei readily disintegrate and the deoxyribonucleohistone dissolves), however, is initially low, and only increases with the breakdown of the cell components. Furthermore, although suspensions of nuclei isolated by certain methods have a low, variable content of deoxypentosenuclease activity, preparations obtained by other methods do not contain the enzyme. These conclusions are in contrast to the findings of Lang, Siebert, Baldus and Corbet (13) that the nuclei isolated from hog and calf kidney by an undescribed "newly developed procedure" contain almost the whole deoxyribonuclease (depolymerase) activity of these tissues. They derive some support, however, from the experiments of Brown, Jacobs and Laskowski (2) in which it was shown that the greater

part of the deoxypentosenuclease activity of calf thymus was contained in the particulate fraction of the cytoplasm (cf. the following paper), while the least activity was associated with the nuclei.[1]

SUMMARY

The deoxypentosenucleases found in homogenates of whole tissue (i.e. calf thymus, rat liver and mouse tumour tissue) are shown to be associated with the cytoplasmic components as separated according to established methods. The low variable deoxypentosenuclease activity of the nuclear fractions isolated by certain procedures is considered to be an artefact resulting from the absorption of the cytoplasmic enzyme during fractionation.

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[1] Since this paper was submitted for publication, Schneider and Hogeboom (*J. Biol. Chem.*, **198**, 155, 1952) have described the results of an investigation of the intracellular distribution of the deoxypentosenuclease activity of mouse liver. In agreement with the present work, their results show that the deoxypentosenuclease of this tissue is confined essentially to the cytoplasm of the cells. Thus 73 per cent of the total enzyme activity of the mouse liver homogenate in 0.25 *M* sucrose was found to be associated with the mitochondrial fraction, whilst nuclei isolated by an improved method of fractionation and which were contaminated with less than 0.5 per cent mitochondria, exhibited less than 1 per cent of the total enzyme activity.

THE PREPARATION AND PROPERTIES OF THE DEOXYPENTOSENUCLEASES OF CALF THYMUS AND MOUSE LEUKAEMIC TISSUE

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Previous investigations of the nature and properties of the deoxypentosenucleases of mammalian cells have been made with unfractionated tissue extracts. Such studies, as discussed by Greenstein, Carter and Chalkley (8), may be influenced by other components of the extracts, and do not necessarily reveal the true properties of the enzymes. In the present work a method has been devised for the isolation and purification of the deoxypentosenucleases of certain tissues. A description of this method, together with a summary of the properties of the purified enzymes forms the subject of the present communication.

EXPERIMENTAL

Measurement of enzyme activity. Deoxypentosenuclease activity was determined viscosimetrically under the conditions described in the preceding paper. In addition to calf thymus deoxyribonucleic acid which was used as the standard substrate, the activities of the purified enzymes were also determined against preparations of the deoxyribonucleic acids from herring-testis, mouse leukaemic tissue and wheat-germ (11) and the deoxyribonucleohistone of calf thymus (7). Except where otherwise stated, the substrates were dissolved in water to give 0.2 per cent (w/v) solutions.

Isolation and purification of the deoxypentosenucleases.

The initial extracts of the tissues were prepared in either 0.2 *M* phosphate buffer saturated with magnesium hydrogen phosphate at pH 6.4, or 0.14 *M* sodium chloride. Under these conditions the cytoplasmic deoxypentosenuclease was readily separated from the insoluble nuclear deoxyribonucleohistone. Both solvents gave equally good results. No disadvantage attended the use of the unbuffered sodium chloride solution, since the tissue extracts themselves had considerable buffering power and maintained a pH favourable for both activity and stability of the en-

zymes. The crude extracts were purified by preferential inactivation of much of the inert protein material followed by fractional precipitation with ethanol. Fractionation of partially purified enzyme solution with ammonium sulphate resulted in considerable losses of activity.

Thymus deoxypentosenuclease. Thymus (400 g), freed from fat and connective tissue, was cooled in ice and homogenized in a high-speed mixer for 2 mins. with the ice-cold solvent (1 500 ml). The resulting suspension was strained through cloth and centrifuged. The turbid supernatant solution (T_1) was covered with toluene (5 ml) and kept for 24–48 hrs. at 4° and then adjusted to pH 5.0–5.2 with 0.1 *N* hydrochloric acid. The heavy white precipitate which separated was immediately removed (centrifuge) and the clear supernatant solution decanted through a plug of cotton wool to remove lipid material. The filtrate (T_2) was adjusted to pH 6.4 with 0.1 *N* sodium hydroxide and dialysed for 24 hrs. against distilled water (10 l) at 4°. The solution was then centrifuged to remove the precipitate which formed on dialysis, and the clear supernatant (T_3) concentrated by pervaporation in cellophane dialysis tubes ($\frac{3}{4}''$ diam.) to c. 1/10th of the initial volume (c. 16 hrs.). The concentrated enzyme solution was centrifuged to remove the slight precipitate which formed during the pervaporation, and the supernatant solution (T_4, pH 5.2) immersed in a water-bath at 35° for 10 mins. This resulted in the separation of a heavy, flocculent precipitate which was removed by centrifuging. The clear supernatant solution (T_5) was dialysed for 48 hrs. at 4° against three changes of distilled water (total vol. 20 l). The precipitate which formed on dialysis was centrifuged off and discarded. The residual solution (T_6) was cooled in a freezing mixture and ethanol at —10° (1/6th vol.) added (final ethanol concentration = 17 per cent (v/v)). During the addition of the ethanol, the temperature of the enzyme solution was brought to —8°. The resulting precipitate (T_7) was separated by centrifuging at 0° and the supernatant solution returned to the freezing mixture. Further precipitates (T_8, T_9 and T_{10}) were collected in this way at ethanol concentrations of 29, 37.5 and 50 per cent (v/v).

The fraction (T_9) containing the greater part of the deoxypentosenuclease activity was dissolved in distilled water (c. 40 ml) and dialysed for 6 hrs. against distilled water (1 l) at 4°. The solution was then diluted to 200 ml and refractionated with ethanol under the above conditions. The active fraction (T_{11}) which separated at an ethanol concentration of 37 per cent (v/v), was suspended in water and dialysed to remove ethanol. This solution was then dried in the frozen state to yield a pale cream coloured powder (20 mg, N 15.8 per cent) which had 750 deoxypentosenuclease units of activity per mg nitrogen.

The various stages in the fractionation procedure are summarized in Table I. The results show that the activity of the initial extract (T_1) was considerably increased by the preliminary acid treatment. Thus, the resulting solution (T_2) had approximately thirty times the activity of T_1. If this step in the purification was omitted, much of the enzyme activity was lost by precipitation during dialysis. It is possible, therefore, that the deoxypentosenuclease was either initially associated with some particulate

TABLE I

Purification of Thymus Deoxypentosenuclease.

Procedure	Fraction	Deoxypentosenuclease activity	
		Units/mg N	Total Units
0.14 *M* NaCl extract of thymus (400 g)	T_1	–	800
Inert material removed from T_1 by precipitation at pH 5.0–5.2	T_2	–	25 600
Inert material precipitated from T_2 by dialysis	T_3	–	24 800[1]
T_3 concentrated by pervaporation	T_4	–	27 000
Preferential denaturation of inert proteins in T_4 at 35° and at pH 5.2	T_5	10	13 200
Purification of T_5 by dialysis	T_6	50	9 000
Fractionation of T_6 with ethanol at −8°			
Ethanol concentration 17 per cent	T_7	50	1 600
» » 29 » »	T_8	270	2 400
» » 37.5 » »	T_9	500	3 400
» » 50 » »	T_{10}	180	1 000
T_9 refractionated with ethanol as above. Active precipitate collected at an ethanol concentration of 37 per cent	T_{11}	750	2 300

material of the cytoplasm, or occurred within the cells in a partially inhibited state.

Deoxypentosenuclease from mouse leukaemic tissue. The tumour tissue (c. 35 g) from 35–40 mice, was ground with sand in a mortar for 3–4 mins. and then extracted with 0.14 *M* sodium chloride (100 ml). The extract (L_1, total activity c. 25 units) obtained after centrifuging the tissue suspension, was fractionated according to the method developed for the thymus tissue. After concentration by pervaporation, the enzyme solution (L_4, total activity c. 75–85 units) was kept at 35° for 10 mins. to denature inert protein material, centrifuged and then dried in the frozen state. The crude products thus obtained from three batches of the tumour tissue were combined, dissolved in water (30 ml) and the resulting solution fractionated with ethanol at —8°. The precipitate collected between ethanol concentrations of 29 and 37 per cent was dissolved in water (10 ml), dialysed for 6 hrs. and the solution then evaporated in the frozen state to yield a white powder (5 mg, N 14.9 per cent), which had 180 units of deoxypentosenuclease activity per mg nitrogen.

[1] For this and subsequent deoxypentosenuclease assays, the (dialysed) enzyme solution was made 0.14 *M* with respect to sodium chloride before the activity determinations.

Properties of the deoxypentosenucleases.

The purified deoxypentosenucleases from thymus and the mouse leukaemic tissue were dissolved in water to give solutions which contained *c.* 2 units of enzyme activity per ml.

The solutions of the purified enzymes were stable at room temperature. Thus, no demonstrable loss in activity occurred when the dilute solutions of the deoxypentosenucleases were kept for 24 hrs at 18°. Crude preparations of the enzymes (i.e. after concentration by pervaporation) were less stable, and lost *c.* 40 per cent of their activity during the same period.

pH optima. The activities of the enzyme solutions (1.0 ml) were measured at different pH values by the formation of "acid-soluble" nucleotides from a substrate of 0.1 per cent thymus deoxyribonucleic acid (2.0 ml) as previously described (7). Phosphorus determinations were made according to Allen's (1) method. The results (Fig. 1) show that the deoxypentosenucleases have optimal activity at pH 5.2–5.3, and are almost inactive at pH 6.5–7.0.

The same pH optimum was found for the thymus enzyme when its activity was determined viscosimetrically at different pH values (Fig. 2).

It is well established that the high viscosity of thymus deoxyribonucleic acid is considerably reduced in acid or alkaline solutions. The critical limits between which the viscosity remains constant are pH 5.6 and pH 10.9 (5, 23). Thus the viscosimetric measurements of deoxypentosenuclease activity in the neighbourhood of the optimum pH are complicated by a partial depolymerization of the substrate in the acid solutions. For this reason, the nucleic acid buffer mixtures used in the above determinations were kept at 35° for 10–15 mins. before addition of the enzyme, and in subsequent experiments, in which enzyme activities were determined viscosimetrically, the measurements were made at pH 5.9.

The results of Fig. 1 show that the activities of the enzymes at a given pH were constant, irrespective of whether acetate, phthalate or citrate buffers were used. In veronal buffers, however, the enzymes were markedly inhibited.

In addition to the purified enzymes, crude preparations of the deoxypentosenucleases from rat liver, mouse sarcoma tissue (cf. the preceding paper) and whole rat embryos (obtained from five rats during the 15th–18th day of pregnancy) also possessed optimal activity at *c.* pH 5, and were almost inactive at pH 7. No evidence was obtained from the activity-pH curves of these crude enzyme preparations of the presence in any of the

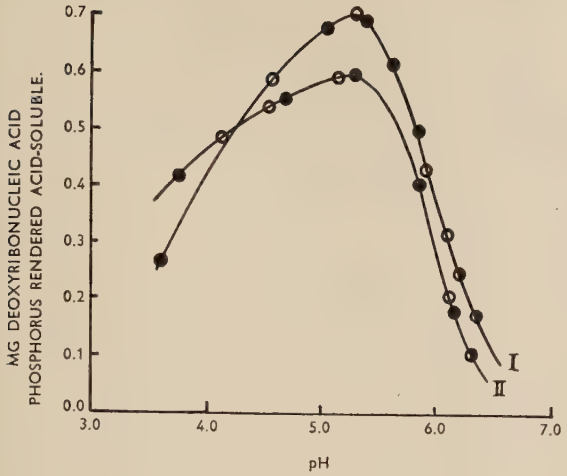

Fig. 1.

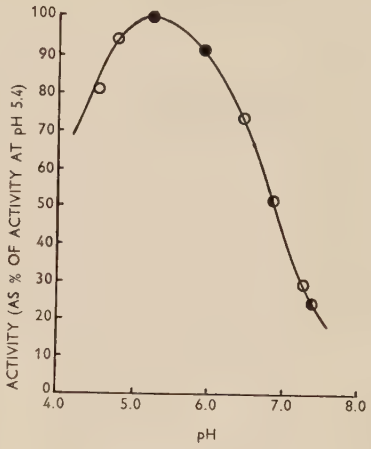

Fig. 2.

Fig. 1. The influence of pH on the activities of the deoxypentosenucleases of calf thymus (I) and mouse leukaemic tissue (II) as measured by the formation of acid-soluble phosphorus. (●) 0.2 *M* acetate buffer, (○) 0.2 *M* phthalate buffer, (◐) 0.2 *M* citrate buffer.
Fig. 2. The influence of pH on the activity of thymus deoxypentosenuclease determined viscosimetrically (○) 0.2 *M* acetate buffer, (●) 0.2 *M* phthalate buffer, (◐) 0.2 *M* citrate buffer.

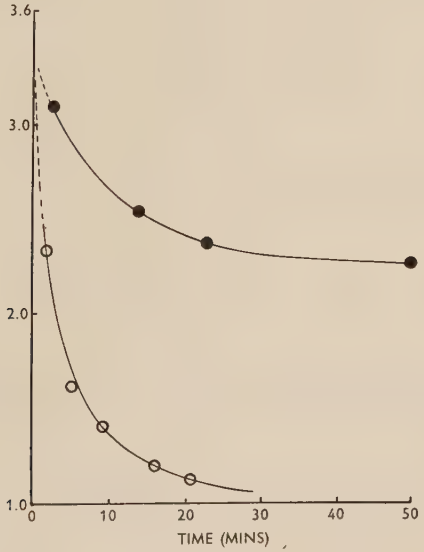

Fig. 3. Activity of calf thymus deoxypentosenuclease at pH 5.9 in the presence of 0.01 *M* (○——○), and 0.1 *M* magnesium sulphate (●——●).

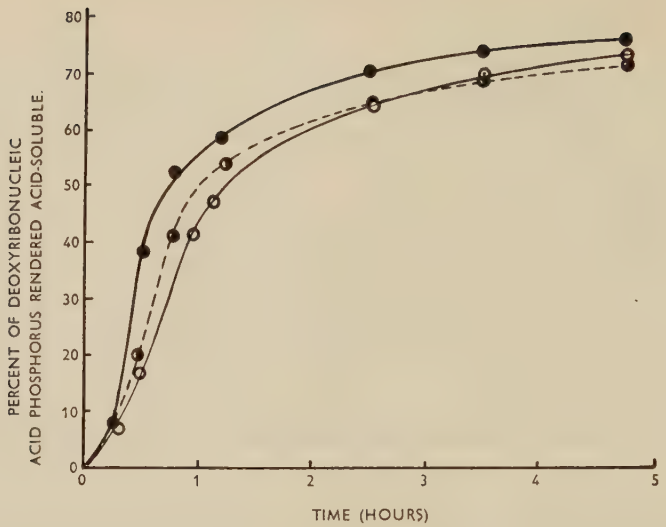

Fig. 4. Action of the purified deoxypentosenucleases from calf thymus (●——●) and mouse leukaemic tissue (◑----◑), and of a crude preparation of the deoxypentosenuclease from rat embryo (○——○), on thymus deoxyribonucleic acid at pH 5.9.

homogenates examined of more than one deoxypentosenuclease. Furthermore, such an enzyme could not be demonstrated by alternative fractionation procedures, as e.g. those described by Zamenhof and Chargaff (22) for the isolation of the deoxypentosenuclease from yeast, and by McCarty (14) for the preparation of deoxyribonuclease from ox-pancreas.

Activation. Solutions of each of the deoxypentosenucleases were inactivated by dialysis. Activity was restored to the solutions by the addition of salts (e.g. NaCl, $MgSO_4$, and cysteine at pH 6). Activity was optimal at a sodium chloride concentration of *c.* 0.06 *M*, and at a magnesium sulphate concentration of *c.* 0.01 to 0.02 *M*. Higher concentrations of sodium chloride (e.g. 0.2 *M*), or, more particularly, magnesium sulphate (0.06 to 0.1 *M*, Fig. 3) inhibited the enzymes.

Cysteine (final conc., 0.03 *M*) at pH 6 activated the thymus deoxypentosenuclease to approximately the same extent as magnesium sulphate at the same concentration and pH. However, the addition of either a mixture of magnesium sulphate (final conc. 0.03 *M*) and cysteine (final conc. 0.03 *M*) or higher concentrations of cysteine alone (i.e. 0.1 *M*) inhibited enzyme activity. The activation of the dialysed enzymes by cysteine, therefore, appears to be an ionic effect and cannot be attributed to the reducing properties of the compound.

TABLE II

Inactivation of the deoxypentosenucleases at 60°.

Mixtures of the enzyme solution (0.3 ml) and buffer (1.7 ml) were immersed in a water bath at 60° for 10, 20 and 40 mins. and then cooled to room temperature. After the addition of 0.1 per cent thymus deoxyribonucleic acid (2.0 ml), the mixtures were incubated at 37° for 5 hrs. and then analysed for "acid-soluble" phosphorus.

Time (mins) at 60	Percent. of Initial Activity			
	Thymus deoxypentosenuclease at		Deoxypentosenuclease from mouse leukaemic tissue at	
	pH 5[1]	pH 7[2]	pH 5[1]	pH 7[2]
0	100	100	100	100
10	52.2	22.2	49.3	11.7
20	29.6	0	23.7	0
40	14.3	0	11.1	0

1. 0.2 *M* acetate buffer.
2. 0.2 *M* citrate buffer.

Heat inactivation. Solutions of the deoxypentosenuclease were readily inactivated at 60°. Inactivation was more rapid at pH 7 than at pH 5 (Table II).

Variation of activity with time. The results (Fig. 4) obtained when the enzyme solution (1.0 ml) was incubated with thymus deoxyribonucleic acid under the previously defined conditions of substrate and enzyme concentration, show that maximum degradation of the substrate to acid-soluble nucleotides occurred in 4–5 hrs. The formation of acid-soluble material showed an initial lag during the first 15 mins. of the experiments and then increased rapidly in the following 30 mins. A similar lag is observed when the action of pancreas deoxyribonuclease on thymus deoxyribonucleic acid is measured by the formation of acid-soluble nucleotides (15) and is attributed to the initial depolymerization of the substrate.

Action on the deoxyribonucleic acids from herring-testis, wheat germ and mouse leukaemic tissue and on thymus deoxyribonucleohistone.

The action of the enzymes on the nucleic acids and the nucleohistone was followed viscosimetrically under the previously defined conditions of enzyme and substrate concentration. There was little difference between the rates of depolymerization of any one substrate by the different enzymes. Representative results (Fig. 5) show that the deoxyribonucleic acid from

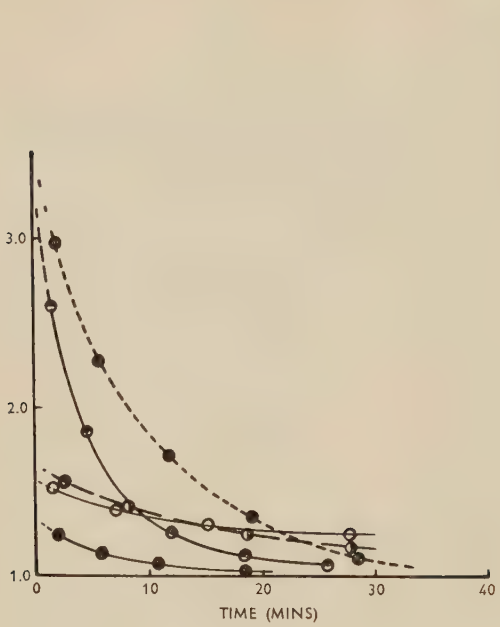

Fig. 5.

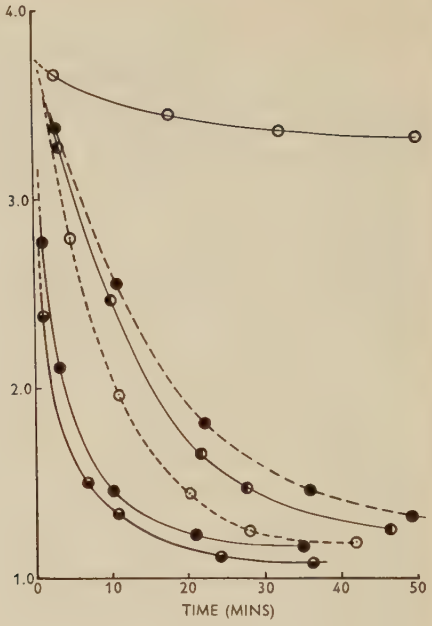

Fig. 6.

Fig. 5. Action of calf thymus deoxypentosenuclease on the deoxyribonucleic acids from mouse leukaemic tissue (◓——◓), wheat germ (●——●), and herring testis (◑ - - - - ◑); and of the deoxypentosenuclease of mouse leukaemic tissue on thymus deoxyribonucleohistone (● - - - - ●) and the deoxyribonucleic acid of herring testis (○——○).

Fig. 6. The depolymerization of thymus deoxyribonucleic acid by calf thymus deoxypentosenuclease alone (○ - - - - ○) and in the presence of 0.05 *M* sodium arsenate (◐——◐) and 0.01 *M* sodium citrate (● - - - - ●); and by the deoxypentosenuclease of mouse leukaemic tissue alone (◒——◒) and in the presence of 0.05 *M* sodium citrate (●——●) and sodium usnate (○——○), see text).

herring-testis was more resistant to enzymic depolymerization than were the other substrates (cf. 11).

Inhibition. Except where otherwise stated, aqueous solutions of the substances examined for inhibitory action were adjusted to *c.* pH 6 with 1 *N* sodium hydroxide or 1 *N* hydrochloric acid and diluted to 0.2 *M* concentration. Various dilutions of these solutions were then prepared in 0.2 *M* acetate buffer, pH 5.9. These precautions were essential since the activities of the enzymes were greatly influenced by both the pH and the ionic strength of the substrate solutions. Method 1: The inhibitor-buffer mixture (1.0 ml) at 34.5° was mixed in the viscometer with the substrate solution (0.2 per cent thymus deoxyribonucleic acid, 1.5 ml) and water (0.5 ml)

at the same temperature, and the enzyme solution (1.0 ml) added. Viscosity measurements were made at frequent intervals during the following 45–60 mins.

Under these conditions, the deoxypentosenucleases were not inhibited by sodium arsenate, sodium borate, sodium citrate, sodium fluoride, sodium selenite, sodium cyanide[1] and iodoacetic acid at concentrations of 0.01 to 0.05 *M*. They were inactive, however, in the presence of zinc and copper ions (0.01 *M*).

Method II: A mixture of a solution of the substance in 0.2 *M* acetate buffer (1.0 ml) at pH 6.0 and the enzyme solution (1.0 ml) was kept at room temperature for 10–15 mins., then warmed to 34.5° and added to the substrate solution (0.15 per cent thymus deoxyribonucleic acid, 2.0 ml) at 34.5°. Saturated solutions (1.0 ml) of hydrogen sulphide[1], chloroacetophenone, colchicine and sodium-*o*-iodosobenzoate[2] in the buffer, as well as aminopterin (4-aminopteroylglutamic acid 2 mg), iodoacetamide (2 mg), sodium ethyl mercurithiosalicylate (1 : 10 000) and hydrogen peroxide (0.5 ml of a 0.6 *M* solution) were without effect upon the activities of the deoxypentosenucleases. The deoxyribonuclease-inhibitor sodium (+)-usnate (1 mg) (12), however, caused almost complete inhibition under these conditions.

Representative results of these experiments, which applied not only to the purified enzymes, but also to the crude deoxypentosenucleases referred to in the previous paper, are shown in Fig. 6.

DISCUSSION

The finding that the intracellular (cytoplasmic) deoxypentosenucleases of various tissues have optimal activity at *c.* pH 5.2 and are almost inactive at neutrality (17), together with the fact that they are not inhibited by various mitotic poisons, suggests that these enzymes are not concerned with the regulation of mitosis. In view of the recent observations that certain cells contain extranuclear deoxyribonucleotides (cf. 9), the cytoplasmic location of the deoxypentosenucleases may prove of some significance with regard to the synthesis and metabolism of deoxyribonucleic acid. However, the acid pH optima of the deoxypentosenucleases suggests that they

[1] These determinations were carried out on half-quantities of the enzyme-substrate mixture in viscometers of the totally enclosed type (10).

[2] Prepared by the oxidation of *o*-iodobenzoic acid according to the method of Askinasy and Meyer (2).

may function in autolysis. This conclusion is in agreement with the results of the fractionation experiments (e.g. Table I) which indicate that the enzymes probably occur within the cells in a partially inhibited state (cf. (22)) and also with the recent observations of Cherry (4) on the cytolysis of mouse liver sections *in vitro*. In the latter investigation it was found that the loss of chromatic material (i.e. the deoxyribonucleohistone) from the nuclei which did not take place until after the dissolution of the nuclear membrane, occurred most rapidly at slightly acid pH values, and was reduced to a minimum at pH 7.4–7.8.

It is probable that the deoxypentosenucleases studied in the present work are identical with those previously described by Greenstein, Carter and Chalkley (8), Maver and Greco (13) and by Brown, Jacobs and Laskowski (3). Thus, in agreement with the present findings, Greenstein *et al.* (8) observed that the thymonucleodepolymerases (deoxypentosenucleases) present in unbuffered aqueous extracts of various tissues were inactivated by dialysis. The activity of the dialysed extracts was restored by the addition of salts such as sodium chloride, magnesium chloride and arginine monohydrochloride. Optimal activation of the enzymes with magnesium salts occurred at a concentration of 0.012–0.024 *M*, whereas higher concentrations were inhibitory (cf. Fig. 3). The inhibitory action of magnesium salts in concentrations greater than 0.024 *M* probably explains the findings of Maver and Greco (13) that 0.01 *M* magnesium sulphate caused partial inhibition, and 0.03 *M* almost complete inhibition of crude preparations of the deoxypentosenucleases of calf thymus and spleen, since these enzyme solutions, which had optimal activity at pH 4.5 already contained sufficient salts for activation.

It is apparent that the properties of the intracellular deoxypentosenucleases of the different tissues examined, while closely similar among themselves, differ appreciably from those of pancreas deoxyribonuclease. In particular the latter enzyme, which has optimal activity at pH 7–8, and which requires magnesium or manganese ions for activation (6, 14, 15), is inhibited by sodium citrate, sodium fluoride, sodium arsenate and sodium borate (7), whereas the deoxypentosenucleases are not inhibited by these substances. It follows, therefore, as discussed elsewhere (21), that the advantages claimed for the use of buffered (pH 7) aqueous solvents containing low concentrations of sodium arsenate, sodium citrate or sodium borate (16, 18, 19, 20) in the isolation of deoxyribonucleic acids are not due to the direct inhibition of the intracellular deoxypentosenucleases, but to the maintenance of a pH unfavourable for activity of these enzymes.

SUMMARY

Partial purification of the cytoplasmic deoxypentosenucleases isolated from homogenates of calf thymus and mouse leukaemic tissue has been achieved by preferential denaturation of much of the inert protein material followed by ethanol fractionation of the concentrated (pervaporated) enzyme solutions.

The purified deoxypentosenucleases have optimal activity at *c.* pH 5, and are almost inactive at neutrality; they are also inactivated by dialysis. Low concentrations of metallic ions (e.g. Na^+ Mg^{++}) restore activity to the dialysed enzyme solutions, while higher concentrations of these ions are inhibitory.

A number of mitotic poisons, and many substances which inhibit the action of pancreas deoxyribonuclease, have no effect on the activities of other deoxypentosenucleases.

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ELECTRON MICROSCOPY OF ISOLATED CHROMOSOMES[1]

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The organization of chromosomes has been the subject of considerable investigation over the years and the knowledge of chromosome structure has been advanced through a variety of experimental approaches. As a result, the gross morphological features of mitotic and meiotic chromosomes have been well defined. However, structural details in the range of 0.01 to 0.2 microns are not well known. To advance the knowledge in this region the electron microscope has been used by a number of investigators on chromosomes prepared in a variety of ways. Pease and Baker (8) have examined ultrathin sections of salivary chromosomes from *D. melanogaster*. Schultz, MacDuffee, and Anderson (9) have developed a smear technique for the preparation of thin samples of animal chromosomes and have examined smears of salivary chromosomes from *D. melanogaster*. A replica technique has been used by Palay and Claude (7) for the electron microscopic examination of the above chromosomes. Hovanitz (4) has prepared isolated chromosomes from chicken erythrocyte nuclei by the method of Mirsky and Ris (5) and has published electron micrographs of the isolated chromosome strands. Denues (2) carried the work of Hovanitz one step farther and examined isolated chromosome strands which had been treated with 1 M sodium chloride solution in order to remove an outer layer of nucleohistone. Recently, Hoffmann-Berling and Kausche (3) have published electron micrographs of chromosomes isolated from chicken erythrocytes and various bovine tissues. In these micrographs they have identified longitudinally aligned fibers in the chromosome strands and have pointed out the recurring dark bands, or disks, resembling the well known structure of the giant salivary chromosomes.

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In the present investigation (10) chromosomes have been isolated from turkey and chicken erythrocytes and from the thymus and liver of the calf by several isolation procedures. Electron microscopy has been used in conjunction with certain chemical and enzyme treatments to determine the organization of the chromosomes. Special emphasis has been placed on the use of an electron staining procedure for desoxyribose nucleic acid which has been described by Bretschneider (1).

MATERIALS AND METHODS

Isolation of chromosomes. Chromosomes were isolated from fowl erythrocytes by a slight modification of the procedure described by Mirsky and Ris (5). Oxalated turkey or chicken blood was frozen at —20° C immediately upon withdrawal from the fowl, since it was found that freezing greatly facilitated the rupture of the nuclei in the subsequent Waring blendor treatment. A 175–200 ml portion of frozen blood was allowed to thaw overnight in a cold room at 4° C and all subsequent operations were carried out in the cold room. After separation of plasma by centrifugation, the layer of cells and nuclei was diluted with 200 ml of 0.14 M sodium chloride solution (unbuffered pH 5.7) and filtered through a double layer of washed cheesecloth to remove any clots present. The cells and nuclei were collected by centrifugation and washed with 175 ml of saline by thorough mixing in the centrifuge tubes. The washing and centrifuging process was repeated six times, until the supernatant liquid was practically colorless and the residue of cells and nuclei was light tan in color. At this stage the residue of cells and nuclei was equally divided among six centrifuge tubes. Since the number of leucocytes found on the surface of the cell layer in the first centrifugation was exceedingly small no effort was made to remove them at this point. During the washing process just mentioned, the small white residue of leucocytes in the bottom of the tubes after centrifugation was separated by transferring the supernatant erythrocyte nuclei to clean tubes.

The contents of each tube of washed nuclei, diluted with saline to a total volume of 200 ml, was subjected to 20 minutes treatment in the Waring blendor. The aluminum blendor container was fitted with a cooling jacket containing ice-ethanol. The chromosomes were collected by centrifugation and were washed twice by dispersing in saline in the blendor for a few seconds. The collected, washed chromosomes were dispersed in saline with the blendor and were stored in plastic centrifuge tubes in the cold room. The chromosomes were generally used the day following isolation and no chromosomes older than three days were used for any purpose. In order to compare the chromosomes isolated with those shown in the literature, they were stained with aceto-orcein and examined in the light microscope. They appeared identical with the chromosomes of Mirsky and Ris (5). An electron micrograph of a turkey chromosome is shown in Fig. 1. Unless stated otherwise, all work reported in this paper was carried out on chromosomes isolated by the above procedure.

An investigation of the progress of the rupture of nuclei in the blendor was made on specimens stained with aceto-orcein. It was found that after 20 minutes of blending the homogenate was composed largely of chromosomes, together with a few intact nuclei. There was no appreciable increase in chromosome yield beyond the 20 minute blending time recommended by Mirsky. The process of releasing chromosomes from nuclei is a gradual one during the blending period used. The effect of dilution on the chromosome yield was also studied and a rather dilute suspension found to give a larger yield of chromosomes. The dilution used in the present work was based on this finding.

Chromosomes were also isolated from turkey erythrocyte nuclei by ultrasonic treatment of a saline suspension of nuclei prepared as mentioned above. A model U-300 Ultrason generator was used. A 20 minute exposure at 2 kv and 200–230 ma produced approximately 85 per cent rupture of nuclei, as determined by examination of the material, stained with aceto-orcein, in the light microscope. The chromosomes were collected by centrifugation and washed twice with saline.

Thymus lymphocyte chromosomes and liver chromosomes were isolated from calf tissue as described by Mirsky and Ris (6) except that the Waring blendor was substituted for the colloid mill for resuspending the chromosomes.

Electron microscope technique. The RCA, Model EMU electron microscope was used for the examination of isolated chromosomes. The chromosome preparations were mounted on Formvar films supported by stainless steel screen. A drop of chromosome suspension was placed on the Formvar covered screen and 5 minutes allowed for solid material to settle. After washing the residue with water, staining with acid reagents (chromic, phosphotungstic, molybdic or osmic acids) was carried out. In the case of aceto-orcein, equal volumes of chromosome suspension and dye solution were allowed to stand 5 minutes before placing a drop of the suspension on the screen.

Chromium shadowing was carried out on certain chromosome samples using object to shadow ratio of 1 to 3 and 1 to 5.

Chemical and enzyme treatment of isolated chromosomes

1 M sodium chloride. Chromosomes isolated from turkey erythrocyte nuclei were treated with 1 M sodium chloride, as described by Denues (2), in order to remove an outer layer of nucleohistone on the chromosome. The resulting "residual chromosomes" when shadowed with chromium, with and without chromic acid fixation, gave structures similar to those reported by Denues.

Strands which appeared identical to the above "residual chromosomes" were obtained by similar 1 M sodium chloride treatment of turkey erythrocyte nuclei.

Bretschneider staining procedure. The procedure described by Bretschneider (1) for the demonstration of desoxyribose nucleic acid in bull sperm has been applied in the present investigation to whole and "residual" chromosomes from fowl erythrocytes and whole chromosomes from calf thymus and liver.

One tube of chromosome suspension which had settled by standing in the cold room overnight was decanted to remove most of the saline and leave a residue of about 10 ml. The use of chromosomes which have been centrifuged and then dispersed by stirring is not desirable because the material remains clumped. Three

ml of chromosome suspension was used for each experiment. The procedure followed is given below and the stipulated times were carefully observed.

1) *Mercuric chloride fixation.* To the chromosome suspension in a 15 ml graduated centrifuge tube was added 6 ml of saturated mercuric chloride solution and 3 ml of absolute ethanol. The tube contents were mixed by pouring back and forth into another tube and by stirring with a platinum rod. The tube was allowed to stand one hour at room temperature. After centrifuging briefly in a clinical centrifuge to collect the solid, the liquid was decanted.

2) *Alcoholic iodine.* To the residue from 1) was added 3 ml of alcoholic iodine solution, mixed as in 1) and allowed to stand one hour at room temperature. The solid was collected and liquid decanted as in 1).

3) *Dimedon.* To the residue from 2) was added 3 ml of 1 per cent dimedon in 90 per cent ethanol, mixed as in 1) and allowed to stand 24 hours at room temperature. The solid was collected and liquid decanted as in 1).

4) *Alcohol sequence.* To the residue from 3) was added 7 ml of 80 per cent ethanol, mixed by stirring and centrifuged briefly. To the residue was added 7 ml of 70 per cent ethanol and the process repeated through 60, 50, 40, 30, 20, 10 per cent ethanol to water. The residue was separated by centrifugation.

5) *Hydrochloric acid hydrolysis.* The residue from 4) was mixed with 7 ml of 1 N hydrochloric acid and the mixture heated for 12 minutes at 55° C in a water bath. The hydrochloric acid was decanted and the residue washed with water, using the centrifuge, until the test for chloride ion with silver nitrate was negative.

6) *Silver nitrate staining.* To the residue from 5) was added 7 ml of silver nitrate, which had previously been treated with 8 per cent ammonium hydroxide (3–4 drops to 55 ml of 10 per cent silver nitrate solution) and filtered through quantitative filter paper. The ammoniacal silver nitrate solution was always prepared immediately before use. The mixture of chromosomes and silver nitrate was heated in a water bath in the dark at 40° C for 24 hours. The residue was separated and washed 5 times, as before, with water containing a few drops of 8 per cent ammonium hydroxide.

7) *Pepsin digestion.* To the residue from 6) was added 7 ml of 1 per cent pepsin solution in 0.1 N hydrochloric acid and the mixture heated at 40° C, as above, for 24 hours. The residue was washed 5 times with water. The residue suspended in water was used for preparation of specimens.

In the original procedure (1) the silver nitrate solution was stated to be pH 8–9, obtained by addition of 8 per cent ammonium hydroxide. It was found in the present work that 10 per cent silver nitrate solution had a pH of 8.9 at 20° C. Addition of one drop of 8 per cent ammonium hydroxide to 55 ml of 10 per cent silver nitrate solution gave a pH of 10.2; 3 drops, 10.2; 4 drops, 10.3. The use of a larger amount of ammonium hydroxide was avoided because silver oxide precipitated and completely obscured the electron microscope specimens.

The results of the Bretschneider procedure applied to whole chromosomes from fowl erythrocytes are shown in Fig. 2 and 3; similar treatment of "residual chromosomes" is shown in Fig. 4, 5, and 6. The application to calf thymus chromosomes is shown in Fig. 7 and 8. The results on calf

liver chromosomes were, in general, similar to those from thymus but the low yield of chromosomes from this source limited further investigation.

As a control, sheep erythrocytes, isolated from both freshly drawn and frozen blood by the procedure described for fowl erythrocytes, were subjected to Waring blendor treatment and the Bretschneider staining procedure. Although occasional chromosome-like structures were seen in specimens stained with aceto-orcein, the characteristic beaded strands formed from true chromosomes by the Bretschneider procedure were completely absent. A sample of the sheep cells was subjected to fractional centrifugation in saline to remove leucocytes. The purified erythrocytes gave similar negative results with respect to the presence of chromosomes.

In order to determine that the tubule evident in the figures showing the results of the Bretschneider procedure is not an artefact, the fixative, staining and digestion processes used in this procedure were studied. Other fixatives, namely 0.025 M barium chloride and Flemming's fluid, gave results similar to mercuric chloride (i.e., beaded strands) when substituted for it in Step 1. Fixatives such as Carnoy's fluid, neutral formalin and 2 per cent lanthanum acetate produced only indefinite masses. Since the beaded strands were few in number and were associated with much material of indefinite structure when barium chloride and Flemming's fluid were used, it was concluded that mercuric chloride is the best fixative for the preservation of unagglomerated chromosomes. Elimination of various steps in the Bretschneider procedure applied to turkey erythrocytes, namely 1, 2, 3, 5 and 6, one at a time, produced only indefinite masses of material. Attempt to break up the material by exposure to ultrasonic vibrations was unsuccessful. Omission of Step 7 resulted in uniformly stained chromosomes with no evidence for localization of desoxyribose nucleic acid (see Discussion following). The use of 0.1 N hydrochloric acid alone, without pepsin, in Step 7 revealed tubules. When trypsin was substituted for pepsin all evidence of structure was destroyed and only finely divided particles were seen in the electron microscope. Ribonuclease and desoxyribonuclease produced agglomerated masses.

Preliminary chemical analyses of the dried tubules from the Bretschneider procedure applied to turkey erythrocytes showed approximately 2 per cent phosphorus, 4.87 per cent nitrogen, 7.17 per cent silver and negligible amounts of ribose and desoxyribose.

Turkey erythrocyte chromosomes mounted on silica films over Formvar coated electron microscope screen were treated with 5 per cent trichloroacetic acid for 15 min. at 90°. They were then stained with 0.2 M lantha-

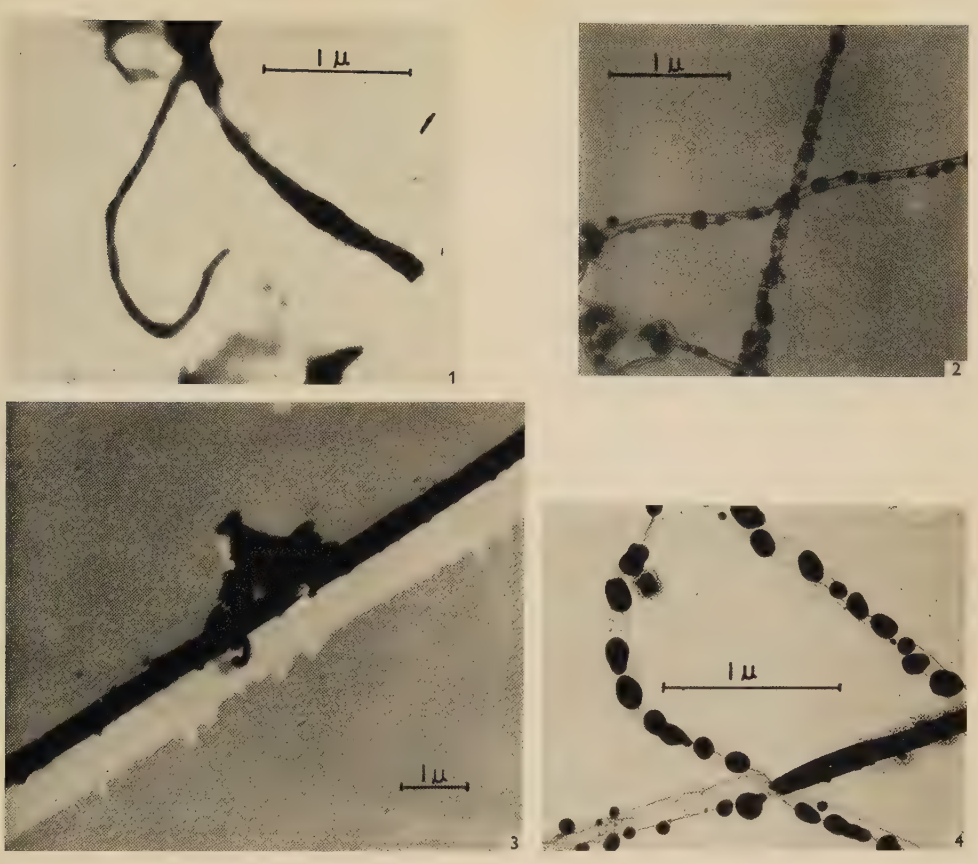
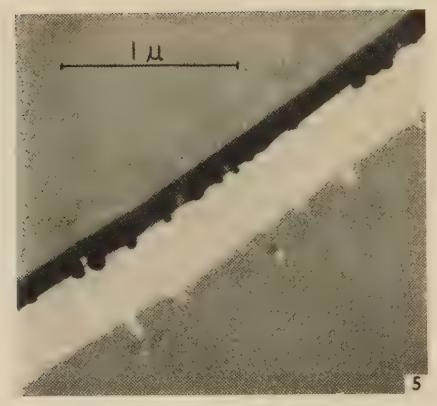


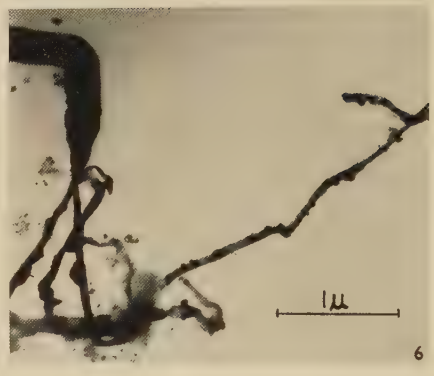
Fig. 1. Turkey chromosome stained with aceto-orcein.
Fig. 2. Chicken chromosome after Bretschneider staining procedure.
Fig. 3. Turkey chromosome after Bretschneider staining procedure, shadowed with chromium.
Fig. 4. Turkey chromosome after 1 M NaCl treatment. Bretschneider staining procedure.

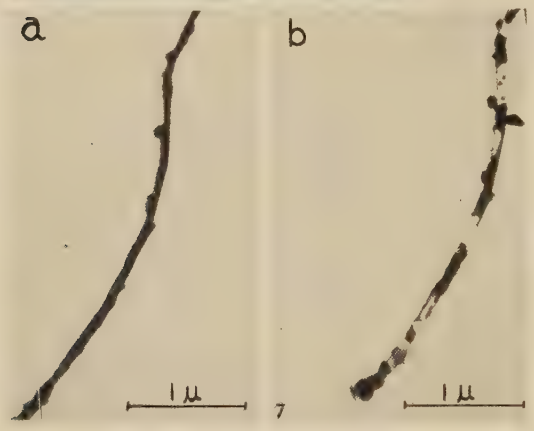
num acetate. Examination in the electron microscope revealed strands in a network formation similar to some of the illustrations of Hoffmann-Berling and Kausche (3). None of the internal structure of the chromosome was revealed.

DISCUSSION OF RESULTS

Whole chromosomes from fowl erythrocytes, when stained with aceto-orcein or with the electron stains mentioned, were found to be moderately kinked strands with diameters ranging from 0.06 to 0.5 micron and lengths







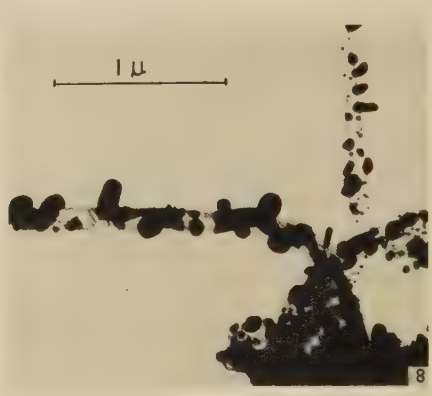


Fig. 5. Same as (4), chromium shadowed.
Fig. 6. Same as (4), showing unravelling.
Fig. 7. Calf thymus chromosome after Bretschneider staining procedure. (a) Observation at low beam intensity: (b) after exposure to high beam intensity.
Fig. 8. Calf thymus chromosome after modified Bretschneider staining procedure—0.1N HCl substituted for pepsin.

from 3–8 microns. The light micrographs of Mirsky and Ris (5) show a range of diameters from 0.3 to 0.8 micron and lengths from 3 to 5 microns. There was no definite evidence for chromomeres or multiple strands when studied by the above techniques.

"Residual chromosomes" from turkey erythrocytes when shadowed with chromium, with and without chromic acid fixation, gave structures similar to those reported by Denues (2). The reduction in diameter due to 1 M sodium chloride treatment is notable and the "residual chromosomes" were thus found to be kinked strands with an average diameter of 0.05

micron. There was no evidence for the association of two or more strands in most of the structures observed.

Typical results of the silver staining procedure of Bretschneider (1) applied to fowl erythrocytes and calf thymus (Fig. 2–8) show that material of high electron scattering power, probably silver, is distributed along the chromosome strands. The uniform distribution observed at low beam intensity (Fig. 7 a) changed upon exposure to higher beam intensity, and staining material was observed to leave certain regions either by migration or evaporation to give the structure seen in Fig. 7 b. Chromium shadowing indicated that the strands were not flat ribbons but had cross-sections that were approximately circular. The dark projections protruding from the strands were present before exposure to the electron beam since the projections cast shadows (Fig. 3, 5). A marked stiffening of the chromosome strands after the Bretschneider procedure has been observed. The frequent curved form of the chromosome strands seen in Fig. 1 has entirely disappeared and the stiff strands occasionally project vertically from the specimen film. The specific agent in the procedure which causes this rigidity has not been determined. The principle feature of chromosome structure revealed by these illustrations of the Bretschneider procedure is the long thin cylindrical structure which will be called a tubule. Although various sizes of strands have been found, completely identical structures were obtained by this procedure, regardless of whether the starting material was whole chromosomes from fowl erythrocytes and from calf thymus or "residual chromosomes" from turkey erythrocytes. Likewise, chromosomes isolated by ultrasonic treatment of turkey erythrocyte nuclei gave structures identical with those from the Waring blendor treatment. In many cases longer strands were obtained by ultrasonic treatment.

The illustrations of Hovanitz (4) show none of the internal structure of the chromosomes shown in the present work, although the presence of protuberances from the central core of the chromosome was observed. The coiled structure observed by Hovanitz has also been shown by Denues. The work of Hovanitz presents a distinct advance over the light microscope results previously reported.

In recent work, Hoffmann-Berling and Kausche (3) have reported the isolation of chromosomes from chicken erythrocytes and from calf thymus and liver by grinding in a glass homogenizer in glycerin and suspension of the chromosomes in sugar solution. The specimens, mounted on the electron microscope film, were fixed in formalin vapor, stained with lanthanum acetate or phosphomolybdic acid and finally shadowed with Fe-

Ni or Cr. Some specimens were extracted with 5 per cent trichloroacetic acid at 90° C. Their micrographs showed the presence of three principal structural elements in the chromosome: 1) a central core built up of a framework of numerous fibrils wound over one another in spirals, 2) a disk formation within the central core similar to that known for the giant salivary chromosomes, and 3) a sheath substance which surrounds the central core of the chromosome.

The fibrils of Hoffmann-Berling and Kausche are of 80–100 A° in diameter and are obviously smaller units than the tubules of the present work, which are 500–2000 A° in diameter. No evidence has been obtained in the present work for the presence of either fibrils or disks in silver-stained chromosomes. The tubules seen here appear to be made up of structural units which are below the resolution of the electron microscope (20–40 A°).

The coiled structure which has been shown by Hovanitz and Denues has not been seen as a regularly occurring feature of chromosome structure in the present work. However, one example of "residual chromosomes" from turkey erythrocytes which had been silver-stained by the Bretschneider procedure was found in which a solid strand was seen to be partially unravelled, revealing at least two tubules at the unravelled end (see Fig. 6). The tubules appeared to be in the form of spirals within the solid strand.

SUMMARY

Chromosomes have been isolated from turkey and chicken erythrocytes and from the thymus and liver of the calf by several isolation procedures. Electron microscopy has been used in conjunction with certain chemical and enzyme treatments to determine the organization of the chromosome. Special emphasis has been placed on the use of an electron staining procedure for desoxyribose nucleic acid which has been described by Bretschneider (1). These treatments revealed that the central core of the chromosome consists of a long thin cylindrical structure which has been called the tubule. The tubule is approximately circular with respect to its cross-section and has occasional projections attached to it. The silver staining procedure results in a uniform deposit of silver within the tubule and only after exposure to beams of strong intensity in the electron microscope does localization of silver occur.

The assistance of Richard Smith in the experimental work on thymus chromosomes is gratefully acknowledged.

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LOCALIZATION OF CARBOHYDRASES AT THE SURFACE OF FUNGUS SPORES BY ACID TREATMENT

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The localization of enzymes at the surface of cells has been reported by several groups of investigators (for literature citations see 1, 3, 8, 11). In all cases the nature of the evidence employed has been indirect, with the possible exception of phosphatase demonstrated by the Gomori technique. While this method is extremely useful in many situations, its value for localization at the cell surface is limited due to the extreme thinness of the plasma membrane. Thus it cannot be used to distinguish between a locus at the external surface of the membrane and one interior to the membrane.

The lack of direct evidence for surface location increases the value of different types of indirect data. A potentially useful technique for certain types of cells is based on the phenomenon of inactivation of certain enzymes of intact cells by exposure to low pH without disruption of cellular metabolism. Many years ago it was known that treatment of yeast cells with acid destroyed their invertase activity, yet did not kill the cells nor interfere seriously with their fermenting properties (16). This evidence was used to support the hypothesis of direct fermentation of sugars, yet it can also serve to localize invertase at the surface of the yeast cells as was subsequently claimed by Wilkes and Palmer (15) and Nelson and Wilkes (10) on the basis of similarities of the pH responses and kinetics of the enzyme *in vitro* and *in vivo*. In a previous paper (8) acid inactivation of an enzyme, in intact *Myrothecium verrucaria* spores, which oxidizes ascorbic acid was reported. These data, together with results of other experiments showing the similarities of the pH activity curves for the enzyme, were used to localize the enzyme at the cell surface. While it was stated that the acid treatment did not affect the viability nor the respiratory activity of the spores, such data were not reported. The present paper reports the results of such experiments. In addition the inactivation of several carbohydrases in spores of *M. verrucaria* and *Aspergillus luchuensis* by acid treatment is demon-

strated. The relation of this type of evidence to the location of the inactivated enzymes is discussed and the conclusion reached that these enzymes are surface located.

METHODS

Spores of *Myrothecium verrucaria* (QM 460) were obtained from cultures grown as described previously (7). Spores of *Aspergillus luchuensis* (QM 873) were grown in similar manner with the exception of using 2 per cent sucrose as carbon source in place of the filter paper. To prepare spore suspensions of *A. luchuensis*, the spores were first wetted with distilled water containing 0.05 per cent Duponal. No wetting agent is necessary to prepare suspensions of *M. verrucaria* spores. In all cases the spores were washed three times by centrifugation and suspended in distilled water.

Acid treatment of the spores was effected by combining equal volumes of spore suspension with 0.2 N HCl and incubating for the indicated time at 30° C on a shaker. The acid treatment was terminated by either centrifuging the spores and washing with 0.05 M $KHPO_4$ buffer at pH 6 or by adding an aliquot of K_3PO_4 known to bring the acid solution to about pH 6. Hydrochloric acid was used in all of the experiments reported here although sulfuric acid works equally well, at least for *M. verrucaria* spores. While nitric acid inactivates the invertase of these spores it also kills the cells.

Carbohydrase activity was measured by adding the appropriate substrate (1 per cent final concentration) to spore suspensions and incubating on a shaker at 30° C. Hydrolysis of non-reducing substrates was followed by determination of reducing sugars at appropriate intervals by the dinitrosalicylic acid method of Sumner (13), and of reducing substrates by determination of glucose with the glucose oxidase method of Keilin and Hartree (5, 6).

Respiration was measured by conventional Warburg technique. Viability was determined by diluting the suspensions and plating out on agar containing sucrose and yeast extract. In all cases where viability determinations were carried out, aseptic precautions were observed. Growth was measured by cell volume determinations in hematocrit tubes after incubation with sugar and yeast extract (9).

RESULTS

Inactivation of the invertase of *M. verrucaria* spores by acid treatment is shown in Fig. 1 a. After continued incubation with sucrose the acid treated spores commence to hydrolize the sucrose, the rate increasing with time. If, however, toluene is added to kill the acid treated cells no resynthesis or reactivation of the invertase occurs. The development of invertase activity after acid inactivation represents resynthesis of the enzyme rather than reactivation after restoration to solutions of normal pH. This point will be discussed in more detail further on.

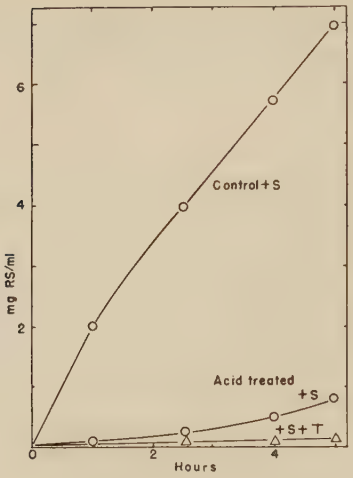

Fig. 1 A.

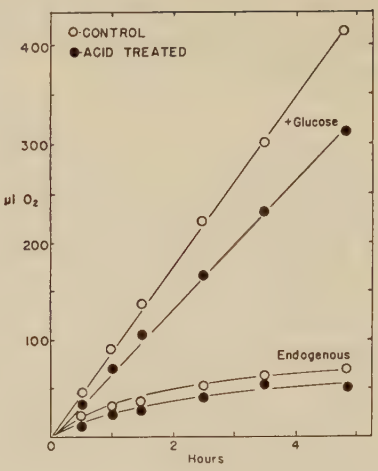

Fig. 1 B.

Fig. 1. Effect of acid treatment on (A) invertase and (B) respiration of *M. verrucaria* spores (20 mins. acid treatment; acid removed by washing; in (A) S = sucrose, T = toluene).

Of critical importance in interpreting this phenomenon of acid inactivation is the effect of the treatment on other physiological processes of the cells. Data show (Fig. 1 b) that neither the exogenous (with glucose) nor endogenous respiration of the spores is decreased greatly. Other data demonstrate that acid treated spores which cannot hydrolyze sucrose respire this sugar at a rate comparable to that for controls. This is ascribed to non-hydrolytic metabolism of sucrose, as will be brought out in a subsequent paper.

To test further the effect of acid treatment on the spores, viability tests were carried out as well as measurements of capacity for growth. Previous studies have demonstrated that spores of *M. verrucaria* swell rapidly in solutions of yeast extract and sucrose, and that the rate of swelling can be used as a measure of growth rate. This swelling is a true growth phenomenon, being accompanied by more or less parallel increases in dry weight and by germination (9). Measurements of invertase activity were run concurrently. The data show (Table I) that while one minute exposure to 0.1 N HCl inactivates almost completely the invertase of the spores, there is no significant effect upon increase in cell volume even after 25 minutes exposure. While the viability appears to have been affected to an appreciable extent by this treatment, the significance of the differences is un-

TABLE I

Effect of acid treatment on invertase activity, viability and rate of growth of *Myrothecium verrucaria* spores.

(Acid neutralized with K_3PO_4).

	Duration of acid treatment	0	1	2	3 hrs.
Invertase activity ..	0 mins.	0	0.20	0.39	0.60 mg R.S./ml
	1 »	0	0	0.10	0.15 » »
	5 »	0	0	0	0 » »
	25 »	0	0	0	0 » »
Cell volume	0 mins.	16	26	41	89 µl
	1 »	16	24	36	82 »
	5 »	16	24	36	82 »
	25 »	16	23	31	84 »
Viability	0 mins.	1.6×10^8 viable spores/ml of susp'n.			
	1 »	1.3 » » » » » » »			
	5 »	1.3 » » » » » » »			
	25 »	1.0 » » » » » » »			

certain. Acid treatment affects the surface properties of the spores. The decrease in viability is considered to be apparent and may be ascribed to some clumping of the treated spores or to their adhesion to the walls of flasks and pipettes during dilution to appropriate concentrations for plating out. The cell volume assay is considered a more reliable measure of viability in this case since the increase in cell volume is a function of the number of viable cells present, and constitutes better evidence attesting to lack of injury to vital metabolic pathways within the cells.

More ideal carbohydrase systems to localize at the spore surface by the acid treatment technique would be the amylases or the cellulases. In these cases the large molecular size of the substrate would in itself tend to exclude penetration into the cell for hydrolytic cleavage. Unfortunately suspensions of intact, viable spores of *M. verrucaria* do not hydrolyze these substrates (starch or cellulose) at measurable rates.

Studies of the metabolism of carbohydrates by *A. luchuensis* spores have shown that they hydrolyze sucrose and cellobiose at rates greatly in excess of their metabolic requirements; maltose and trehalose are hydrolyzed slightly in excess (unpublished). After acid treatment these spores no longer

TABLE II

Effect of acid treatment on hydrolysis of sugars by *Aspergillus luchuensis* spores.

(20′ acid treatment; acid removed by washing; data for sucrose in terms of red. sugar, for other sugars as glucose—all in γ/mg spores/hr.).

Sugar	Treatment	Rate of hydrolysis
Sucrose	Control	116
	Acid treated	7
Maltose	Control	40
	Acid treated	0[1]
Trehalose	Control	24
	Acid treated	0[1]

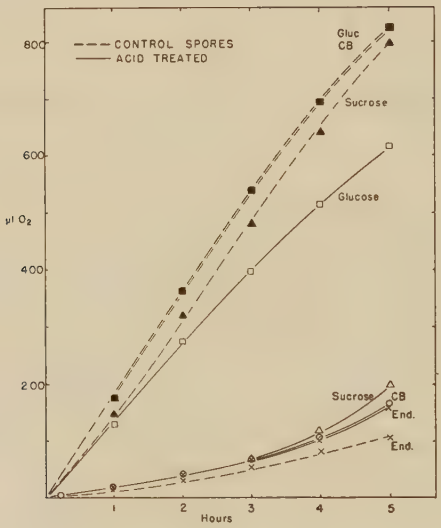

Fig. 2. Effect of acid treatment on endogenous (End.) respiration of *A. luchuensis* spores and on respiration with glucose (Gluc.), cellobiose (CB), or sucrose.

hydrolyze sucrose, maltose or trehalose (Table II). Furthermore, such spores cannot respire sucrose, yet respiration on glucose and endogenous respiration is only slightly affected (Fig. 2). While the cellobiase activity of acid treated spores has not been measured, the inability of such spores to respire cellobiose (Fig. 2) indicates cellobiase inactivation.

[1] No measurable hydrolysis indicates less than 4 γ/mg/hr.

DISCUSSION

In considering the results of this study several basic facts should be pointed out. The enzymes studied here are all present in excess of the metabolic requirements of the cells for the products of enzymatic action. Thus enzymatic activity of the living spores can be measured by analysis of the suspension medium. It is also fundamental that these enzymes are not released from intact cells.

Data presented here have demonstrated the inactivation of the invertase of *M. verrucaria* spores and also of the invertase, cellobiase, trehalase and maltase of *A. luchuensis* spores by brief exposure of the spores to acid. Interpretation of the significance of these data hinges upon a knowledge of what other effects such treatment has upon the cells. Of primary importance in this connection is the establishment of the resistance of the spores used to the acid. Exposure for periods of time greatly in excess of that required for inactivation of the enzymes in question has no appreciable effect upon the rate of germination (as measured by cell volume increase), nor are significant numbers of the spores killed. Furthermore, the respiratory activity of the spores is not greatly impaired except where the substrate is metabolized through the inactivated enzyme. On the basis of this information it would not seem possible that hydrogen ions penetrate the cell to inactivate certain enzymes within the cell. If such were the case, a more fundamental disruption of metabolism should occur. Those enzymes which are inactivated must therefore be at the external surface of the plasma membrane. In a previous study the surface location of invertase in *M. verrucaria* spores was inferred from the pH relation of the enzyme *in vitro* and *in vivo*.

The mechanism of inactivation of the enzymes is unknown. Some unpublished data show, however, that the effect of acid on the cells is not restricted to a simple denaturation of protein. Thus analyses of the acid solution after treatment of the spores show phosphates and nitrogen. Furthermore some carbohydrate would appear to be split off from the cells since autolysis of acid treated spores under toluene results in considerably less production of reducing sugars than controls. It is perhaps significant that removal of the acid by washing the spores or by neutralization yields the same results. In none of the cases studied is the inactivation readily reversible as Herriott (4) found for invertase extracted from yeast. Restoration of activity does occur in cells incubated with sucrose overnight (more

rapidly if yeast extract is added). If the cells are killed by adding toluene or if they are suspended only in buffer, nutrient salts solution, or distilled water, invertase activity does not reappear. Furthermore, the "reactivation" does not occur anaerobically nor at temperatures beyond the growth limits of the organism. Hence it is concluded that the development of invertase activity after acid inactivation is due to resynthesis of the enzyme. Whether this represents total synthesis or merely repair of a critical portion of the enzyme is not known. Certainly further experimentation with this type of material could provide valuable data on the problem of enzyme synthesis.

It is interesting to consider in more detail the possible implications of the localization of enzymes at the cell surface upon our concept of the nature of the plasma membrane and also to evaluate more precisely the meaning of cell surface as the locus of enzymic activity. Davson and Danielli (7) conceive of the plasma membrane as consisting essentially of a double film of lipoid molecules bounded on either side by a proteinaceous layer. In the past our thinking has limited the function of this membrane to its properties of differential permeability which regulate, in a physical manner, the diffusive interchange of materials between the interior of the cell and its environment. The localization of enzymes at the cell surface, the biochemical participation of the membrane in the penetration of phosphate into the cell by formation of an organic phosphate which enters the cell, the suggested phosphorylation of sugars at the membrane, the high degree of specificity for penetration of the erythrocyte by sugars, all point toward an active biochemical role of the plasma membrane in addition to its functions of controlling diffusion. This has been discussed in detail by Rosenberg and Wilbrandt (11) and also by Rothstein *et al.* (17). The protein of the plasma membrane then functions not only by endowing the membrane with certain structural properties, but has an additional, more active role in governing the metabolism of the cell. If we were to accept Virtanen's suggestion that all of the protein of the cell is enzymatic in nature (14), then all of the protein of the plasma membrane has enzymatic properties. Certainly the number and variety of types of surface located enzymes is becoming ever greater.

SUMMARY

1. Treatment of spores of the fungi *M. verrucaria* or *A. luchuensis* with 0.1 N HCl destroys the enzymatic activity of the spores toward certain

substrates yet does not significantly affect the viability of the cells, their subsequent rate of growth, nor their general metabolic activity.

2. It is concluded that the enzymes inactivated by this treatment are at the external surface of the plasma membrane. Such enzymes in *M. verrucaria* spores are invertase, and in *A. luchuensis* spores, invertase, cellobiase, trehalase, and maltase.

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STUDIES ON THE MECHANISM OF JANUS GREEN B STAINING OF MITOCHONDRIA[1, 2]

I. REVIEW OF THE LITERATURE

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Although much has been written about the use of Janus Green B[3] as a supravital stain for mitochondria, the mechanism of the staining reaction is not completely understood. We have therefore undertaken a critical analysis of the mechanism of this staining reaction and several preliminary reports have been published (19, 37, 38). Since many of the studies which have been carried out in the past pertain to our current investigation we have tried to review and correlate these studies in this paper. We have not attempted to review all of the literature on JG-B staining since many aspects of this problem have been adequately covered by a number of investigators (25, 29).

In 1900 Michaelis (45) introduced JG-B as a supravital stain for the cell granules which we now know to be mitochondria. Using JG-B (diethylsafranineazodimethylaniline) Michaelis was able to stain filamentous structures within cells, and he recognized that these structures were similar to the elementary filaments described by Altman in 1890 (2). These filaments were later shown to be identical with the structures which Benda called mitochondria (6).

Although Michaelis specified that only the diethyl derivative could be used, other investigators employed different samples of JG and were unsuccessful in using it as a supravital stain (23). It was not until 11 years later that Bensley (7) reintroduced the use of JG-B. Cowdry further extended the use of this dye as a cytological stain and by studying a number of closely related dyes he was able to determine the specific chemical requirements for supravital staining (22, 23).

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[1] Presented in part at the VII International Congress for Cell Biology, New Haven, Conn., Sept. 8, 1950.

[2] Aided by a grant from the National Foundation for Infantile Paralysis, Inc.

[3] When designating Janus Green, Janus Green B and Janus Green G the abbreviations JG, JG-B and JG-G will subsequently be used.

JANUS GREEN B

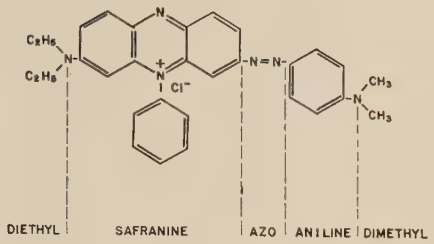


Fig. 1.

THE REDUCTION OF JANUS GREEN B

The structure of JG-B is shown in Fig. 1. This dye is prepared by conjugating diethylsafranine to dimethylaniline through an azo linkage (45). Diethylsafranine is a red dye and JG-B is blue. Thus the conjugation with dimethylaniline alters the resonating structure of the diethylsafranine and shifts the position of the absorption spectrum maximum towards the longer wave lengths. On treating JG-B with reducing agents, the azo bond is usually ruptured irreversibly and diethylsafranine is liberated (9, 45). By using zinc and hydrochloric acid, Bensley (9) was able to show that a reversible reduction product (leuco JG-B) was formed prior to the irreversible rupture of the azo bond. When the zinc was filtered off at a critical stage in the reduction of the dye (when the solution had turned violet) the blue color was regenerated by aeration; i.e. the leuco JG-B was reoxidized to JG-B.

The chemical nature of leuco Janus Green B. The precise chemical structure of leuco JG-B has not been established. From theoretical considerations it is possible that there may be several species of leuco JG-B. If the azo linkage of JG-B were reduced to a hydrazo group, leuco JG-B-I would be formed (see Fig. 2). Since a portion of the conjugated system of double bonds would be destroyed, the color of leuco JG-B-I should approximate that of diethylsafranine. However the color of leuco JG-B-I might be red-violet or purple, since dimethylaniline, when attached to the diethylsafranine, would exert some weighting effect; this weighting effect might produce a slight shift in the absorption spectrum maximum toward the longer wave lengths. If the azine ring of JG-B were reduced prior to the reduction of the azo bond, leuco JG-B-II would be formed. In this case the conjugation in the safranine portion of JG-B would be destroyed, and the resulting azo

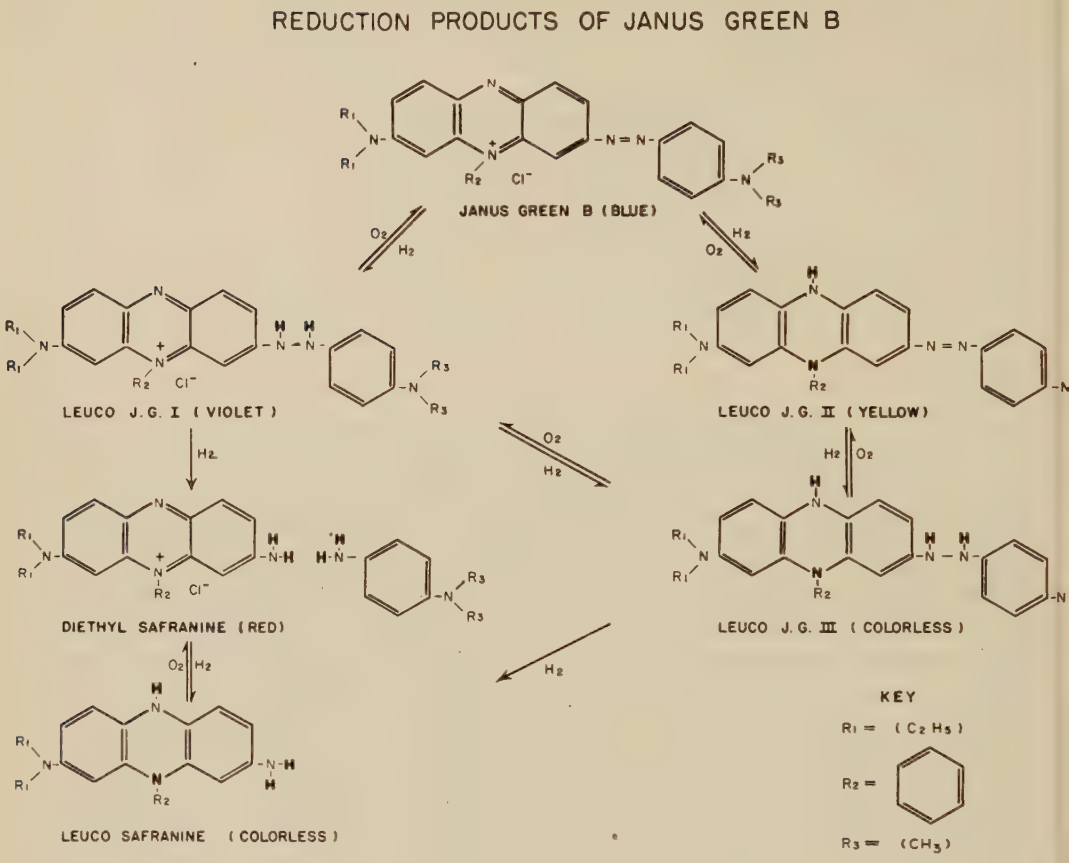
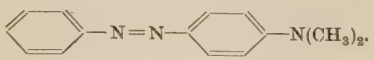


Fig. 2.

compound might have a yellow color similar to p-dimethylaminoazobenzene.[1] If both the azine and the azo linkages were reduced prior to the splitting of the molecule, then leuco JG-B-III would be formed and the resulting compound would be colorless. All three of the postulated forms of leuco JG-B could be reoxidized to the original blue dye. Although a purple color has been observed during the chemical reduction of JG-B by zinc and hydrochloric acid, one cannot necessarily attribute this color to leuco JG-B-I, since a mixture of diethylsafranine and unreduced JG-B would likewise give a purple color.

[1] 

Further reduction of the hydrazo bond of leuco JG-B-I would split off p-aminodimethylaniline with the irreversible production of the red product, diethylsafranine. When the azine ring of diethylsafranine is reduced, a light yellow or colorless derivative, leucosafranine, is formed (see Fig. 2). This last step is reversible and diethylsafranine can be regenerated by bubbling oxygen through the solution (9, 45). Reduction of leuco JG-B-III at the hydrazo linkage would likewise give rise to leucosafranine.

Thus, if leuco compound I were the major intermediate formed in the reduction of JG-B the color would change successively from blue (JG-B) to violet-red (leuco JG-B-I) to red (diethylsafranine) and finally to light yellow or colorless (leucosafranine). Reoxidation (shaking with oxygen) could convert leuco JG-B-I to JG-B and leucosafranine to diethylsafranine but it would not convert the diethylsafranine to leuco JG-B. Thus if leuco JG-B-I is the intermediate, once the dye is reduced to a yellow or colorless form it would not be possible to regenerate the blue color of JG-B. However, if leuco JG-B-II or III were intermediates, a yellow or colorless leuco JG-B would be formed and this could be reoxidized to a blue compound (JG-B) by oxygen. There is some evidence to suggest that a colorless (or yellow) leuco JG-B may be formed within the cell during the biological reduction of the dye (11, 39, 41). This evidence will be considered later.

Enzymatic reduction of Janus Green B. The early studies of Michaelis (45) and Bensley (8, 9) showed quite clearly that the living cell is capable of reducing JG-B, and many other investigators have made use of this fact for a variety of purposes (3, 15, 18, 43). It has been shown that the cell must be alive and healthy in order to reduce the dye (14, 17, 54) and the suggestion has been made (5) that the reduction is enzymatic in nature.

It has also been reported that JG-B is reduced by the lactic dehydrogenase system (4, 10), by a beta hydroxybutyric acid dehydrogenase system (4), and by a formic dehydrogenase system (10).

SELECTIVE STAINING OF MITOCHONDRIA WITH JANUS GREEN B

The staining of mitochondria with JG-B is usually carried out in one of two ways. Either the cells or tissues are placed in a dilute solution of the dye or an entire organ is perfused with an isotonic salt solution containing JG-B.

Staining by organ perfusion technique. When an organ is perfused with a large volume of a 1 : 15 000 JG-B solution, the cells are completely stained. The selective staining of mitochondria develops secondarily as a result of

the differential rate of reduction of JG-B in the various parts of the cell; it is also dependent upon an appropriate oxygen supply (8, 9).

In Bensley's studies on the pancreas (8, 9), guinea pigs were perfused via the abdominal aorta with an isotonic salt solution containing 1 : 15 000 JG-B. When the injection was stopped after 15 minutes, the entire organ was stained a deep blue and there was no evidence of differential staining of mitochondria. When the pancreas was then returned to the abdominal cavity and air excluded by closing the abdominal wall, the tissues reduced the JG-B. After about 15 minutes, when pieces of pancreas were removed and placed under a cover slip, the mitochondria were selectively stained. As the tissue was being studied under the microscope, the central portion of the slide turned pink whereas the peripheral portions of the tissue retained some of the original blue color. The mitochondria in the central portion of the tissue were not selectively stained, i.e. they were colorless or light pink. However, in the cells nearest the periphery, i.e. nearest the oxygen supply, the mitochondria were selectively stained blue. In the acinar cells the mitochondria were seen as blue filamentous structures located in the basal portion of the cell and they could be clearly differentiated from the unstained secretion granules seen in the apical end of the cell. The nucleus and the cytoplasm between the mitochondria appeared unstained. Thus the supravital staining of mitochondria is clearly dependent upon the reduction of the dye under partial anaerobic conditions. Differential staining appeared during the exclusion of oxygen (when the pancreas was returned to the abdominal cavity). The mitochondria in the central part of the tissue lost their stain first presumably because the oxygen was used up and the JG-B was completely reduced. Since oxygen diffuses into the slide from the periphery, the mitochondria in the peripheral portions of the slide remained stained for longer periods, at times for an hour or more. Eventually, however, the mitochondria in the peripheral parts of the slide lost their stain. Decolorization is hastened by rimming the slide with vaseline since this prevents the entrance of oxygen into the tissue section. If the cover slip is removed from a preparation in which the mitochondria have just recently lost their blue color, the oxygen thus introduced reoxidizes the leuco JG-B present and the mitochondria regain their blue color. Eventually, however, all of the JG-B in the mitochondria is reduced to diethylsafranine and leucosafranine, and the mitochondria become red and then colorless. Although Guilliermond and Gautheret (27) have attributed this color change (from red to colorless) to a diffusion of the diethylsafranine out of the mitochondria, it probably results from the reduction of diethylsafranine to leucosafranine. At the leuco-

safranine stage the readmission of oxygen does not restore the blue color (JG-B). When the cell dies the cytoplasm and the nucleus, which may have previously been colorless, become stained red. This color change can be attributed to a loss in the reducing capacity of the cell; the reduction of diethylsafranine to leucosafranine ceases, and the leucosafranine that is present is gradually reoxidized to the red derivative by molecular oxygen.

These results have led other investigators to suggest that the staining with JG-B involves hydrogen transfer (28) and that the specificity stems from the fact that under the proper conditions of oxygen tension, mitochondria reduce the dye more slowly than does the rest of the cell. Thus even though the entire cell may be stained originally, the mitochondria retain their blue color while the dye in the rest of the cell is bleached by reduction (48).

Staining of isolated cells or tissues with dilute solutions of Janus Green B. When a drop of blood is added to a drop of isotonic salt solution containing 1 : 100 000 JG-B, the mitochondria of the white cells become selectively stained whereas the remaining portions of the cell remain unstained (21). These results suggest that the dye is selectively taken up from dilute solution and concentrated in the mitochondria. Thus when a dilute solution of JG-B is used, the intermitochondrial portions of the cytoplasm never appear stained. Partial anaerobiosis may be presumed to exist within the cell and it is probable that the JG-B which reaches the cytoplasm is rapidly reduced. Thus the concentration of unreduced JG-B in the cytoplasm may never be sufficient to be seen. On the other hand if JG-B is reduced more slowly in the mitochondria or if the leuco JG-B formed in the mitochondria or in other parts of the cell were reoxidized in the mitochondria to JG-B, then this structure would become progressively stained.

Effect of the concentration of dye used on the selective staining of mitochondria. The concentration of JG-B used influences the selective staining. Lewis and Lewis (40) found that dilute solutions of JG[1] (1 : 100 000) stain mitochondria almost as intensely as do more concentrated solutions (1 : 5 000). However, with dilute solutions of JG[1] the cytoplasm and nucleus remain clear and the mitochondria are a blue-green, whereas with more concentrated solutions the mitochondria are dark green, but the cytoplasm is pale green, the nucleolus green and the nucleus more or less violet-green. Chambers (13) has also emphasized that JG-B will stain nuclear structures if the dye is used in sufficient concentrations and that the nuclear structures of dead cells will take up the blue stain readily. Presumably when the concentration of the dye is too great, the reducing capacity of the cell is exceeded

[1] The exact structure of the dye was not given, but it is believed to have been Janus Green B.

and differential staining does not take place. Dead cells on the other hand do not reduce JG-B and hence no differential staining takes place.

Cyanide inhibition of Janus Green B staining. Lewis and Lewis (39, 41) were the first to show that cyanide inhibited the JG[1] staining reaction and this fact has since been confirmed by Brenner (11). The addition of .001 M cyanide to supravitally stained cells caused a rapid decolorization of the mitochondria. When the cyanide solution was replaced with fresh Locke's solution, the mitochondria immediately became restained without further addition of the dye. This decolorization and recoloration procedure could be repeated many times. It clearly indicates that leuco JG-B is formed within the mitochondria in the presence of cyanide. These observations suggest that some enzyme system present within the mitochondria normally prevents the reduction of JG-B to its leucobase. When this enzyme is inhibited by cyanide, reduction takes place and the differential stain is lost.

STAINING OF STRUCTURES OTHER THAN MITOCHONDRIA BY JANUS GREEN B

Bensley, in studying the JG-B staining of pancreas in 1911, observed that the islets of Langerhans remained stained for several hours after the mitochondria in the acinar tissues were completely decolorized (8). In fact, this differential staining permitted him to count the total number of islets of Langerhans in the guinea pig pancreas. By examining the islets with the oil immersion lens it was observed that the stain was contained exclusively in small granules which were embedded in a colorless and apparently structureless cytoplasm. When such a preparation was exposed to air, the mitochondria could also be seen in the cell as filamentous structures and these could be differentiated, although with some difficulty, from the specific granulations of the cell which were also stained with JG-B. Eventually the granules in the islets of Langerhans became decolorized.

The kidney glomerulus is likewise stained with JG-B long after the other cells have reduced the dye. Each glomerulus stands out as an intensely blue stained body, whereas the remainder of the tissue is stained red; counting the glomeruli in the kidney is thus possible (47).

JG-B has been used to stain nerve fibers supravitally. Ehrlich (26), as quoted by Michaelis (45), used this dye for that purpose prior to its use by Michaelis (45) as a mitochondrial stain. However the JG-B is quickly re-

[1] The exact structure of the dye was not given, but is believed to have been Janus Green B.

duced to diethylsafranine in the nerve fiber and since the latter dye does not have an affinity for nerve tissue, the selective staining of the nerve fiber is soon lost (46). Couteaux (20) has reported that JG-B selectively stains the myoneural junction. However, a much higher concentration of the dye (1 : 1 000–1 : 10 000) is required to stain the myoneural junction than is the case for mitochondria.

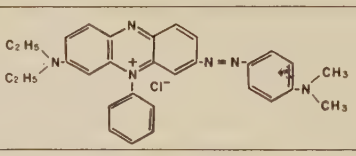
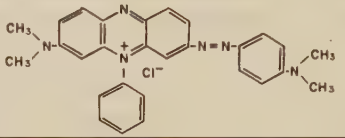
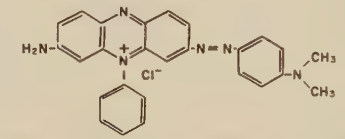
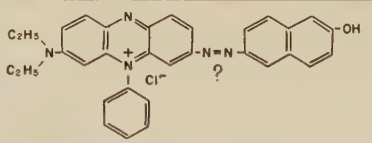
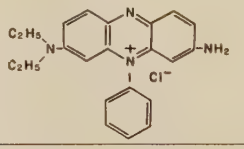
JG[1] has also been used to demonstrate the presence of physiological metabolic gradients in developing embryos (17, 54). Initially the entire embryo is stained blue when it is placed in a solution of JG.[1] However, when the embryos are placed under partial anaerobic conditions, the most active regions of cellular differentiation reduce the dye. These areas are red or colorless and they can be sharply differentiated from the blue background of the embryo. At times, the biochemical differentiation is evident at a time when no morphological evidence of differentiation is visible; the epithelium which is to become the optic cups shows more active reduction of JG[1] than do adjacent parts of the embryo (54).

THE CHEMICAL SPECIFICITY OF JANUS GREEN B AND RELATED DYES IN THE SUPRAVITAL STAINING OF MITOCHONDRIA

Michaelis specified that diethylsafranineazodimethylaniline (JG-B) was required for selective supravital staining of mitochondria (45). Although this dye will stain mitochondria at a dilution of 1 : 500 000 (23), slight alterations of the safranine portion of the dye, such as the substitution of two methyl groups for the two ethyl groups, abolishes the selectivity (see Fig. 3) (23, 45). Dimethylsafranineazodimethylaniline (JG-G) in a concentration of 1 : 10 000 does not stain mitochondria selectively since other specific granulations of the cell are stained more intensely than are the mitochondria. Likewise the substitution of two hydrogen atoms for the two ethyl groups abolishes the specificity; safranineazodimethylaniline (JG) in a concentration of 1 : 10 000 only tinges mitochondria slightly. On the other hand, considerable alterations in the non-safranine portion of the JG-B molecule do not abolish its specificity. For example in Janus Blue a beta naphthol ring is conjugated to diethylsafranine presumably through an azo linkage; this compound stains mitochondria at a dilution of 1 : 200 000 (22, 23). Likewise diethylsafranine monocarboxylic acid[2] (prepared by hydrolysis of the

[1] The author did not specify which of the various types of Janus Green was used.
[2] Exact structure not given.

CHEMICAL SPECIFICITY OF MITOCHONDRIAL STAINING *

DYE	STRUCTURAL FORMULA	LOWEST CONCENTRATION WHICH STAINS MITOCHONDRIA	COMMENTS
JANUS GREEN B		1:500,000	SPECIFIC STAIN OF MITOCHONDRIA ONLY.
JANUS GREEN G		1:200,000	STAINS SPECIFIC WHITE BLOOD CELL GRANULES MORE INTENSELY THAN MITOCHONDRIA.
JANUS GREEN		1:10,000	TINGES MITOCHONDRIA ONLY.
JANUS BLUE		1:200,000	LIKE JANUS GREEN B, BUT STAINS MORE SLOWLY.
DIETHYL SAFRANINE		1:10,000	

* ADAPTED FROM COWDRY (1918)

Fig. 3.

nitrile of JG-B) stained the mitochondria of paramecia in 20 minutes when the dye concentration was less than 1 : 5 000 (exact concentration not given) (34). Diethylsafranine itself will also stain mitochondria selectively if it is used in sufficiently high concentration (23, 59). At a dilution of 1 : 100 the mitochondria are stained intensely red whereas the nuclei of the cell are stained diffusely; at a dilution of 1 : 10 000 the mitochondria stain faintly (23). Although other investigators have stated that diethylsafranine will not stain mitochondria (45, 27) this apparent discrepancy may be due to the use of different concentrations of dye. Cowdry found that staining of mitochondria required about 50 times as much diethylsafranine as JG-B (23).

Thus the diethylsafranine portion of the JG-B molecule is essential for

supravital staining. Since diethylsafranine is more basic than is dimethylsafranine or safranine, Cowdry has suggested (23) that this basic character may play a role in the staining reaction. Conjugation of diethylsafranine with azodimethylaniline or with beta napthol greatly intensifies the staining reaction.

THE ROLE OF PRECIPITATION AND ADSORPTION PHENOMENA IN JANUS GREEN B STAINING

It has been suggested that JG-B must be taken up from solution and concentrated within the mitochondria, for staining has been observed when very dilute solutions of the dye (1 : 500 000) are used (23, 24). There is some evidence to suggest that mitochondria have a limited ability to take up JG-B since supravitally stained mitochondria, once they have become decolorized, do not restain when additional dye is added (23). On the other hand, it has been reported that the dye can be removed from the cell by washing (13).

JG-B forms insoluble precipitates with a variety of biological agents. Lecithin (57, 60)[1] precipitates with JG-B over a very narrow range of dye concentration; hemoglobin (60)[1], normal rabbit serum (60)[1], gelatin (58), typhus bacilli (60)[1] and bacteriophage (61)[1] all form insoluble precipitates with JG-B. The degree of combination of JG-B with gelatin increases with increasing pH (58).

Safranine, a component of JG-B, forms insoluble complexes with trypsin (33, 42, 53), pepsin (42), and with the proteolytic enzymes of *B. proteus* (61). All of the trypsin is removed from solution by safranine and at least 70 per cent of the original proteolytic activity can be recovered in the precipitated complex (33, 42). In contrast to the red color of safranine, a water suspension of the safranine-trypsin complex has a violet color. This reaction with protein is believed to take place through the azine ring of safranine because the reduced form of the dye (leucosafranine) will not form a precipitate with trypsin. Since JG-B also contains an azine nucleus, it has been suggested that JG-B is adsorbed on the mitochondria through the azine nitrogen (42). However, Marston's interpretation (42) that the adsorption of JG-B results from a localization of proteolytic enzymes within mitochondria cannot be accepted.

The combination of JG-B with the mucoprotein secreted by planaria and other invertebrates (23) is believed to interfere with the JG-B staining of these organisms, presumably because the mucous offers an effective barrier

[1] The author did not specify which of the various types of Janus Green was used.

against the entrance of the dye into the cell. Strong salt solutions (3 per cent sodium chloride or potassium nitrate) are reported to interfere with the JG-B staining reaction (23), and it seems possible that high salt concentrations may interfere with the combination of JG-B with proteins.

TOXICITY OF JANUS GREEN B

While the toxicity of JG-B for the living cell can be attributed to its combination with protein, JG-B also inhibits a number of enzymatic reactions such as oxidation of glucose, lactate, succinate, formate (50)[1], fumarase (49)[1], cholinesterase (52)[1], DPN destruction (44)[1], and oxidative phosphorylation (12, 30[1], 35).

The Lewises observed that when JG[2] in dilutions as great as 1 : 200 000 was added to cells it produced cell death within a few hours. In the unstained living cell, as seen in tissue culture, mitochondria normally change their shape continually. They bend and twist; the filamentous structures transform into rods or break up into granules; the granules fuse and form filaments. In cells that are supravitally stained with JG[2] the mitochondrial rods usually break up into granules. In some cases, however, the filamentous structures were preserved and mitochondria which were distinctly stained with JG[2] continued to move about in the cytoplasm and to change their shape. In some of the heart muscle cells in which the mitochondria were stained with 1 : 100 000 JG[2], the muscle continued to beat for 105 minutes. At the end of this time the JG[2] faded out of the mitochondria and the muscle ceased to beat (40). Trypanosomes retained their motility for some time even though the mitochondria were supravitally stained with JG-B (59). Supravitally stained polymorphonuclear leucocytes retained their amoeboid motion, engulfed foreign particles, and showed no deviations from their normal behaviour for 1/2 to 1 hour; the rate of disintegration of these cells was not greatly accelerated by the presence of the stain (23). On the other hand when Hydra were placed in dilute solution of JG[1] (1 : 50 000) they began to disintegrate after about two hours (16). Invertebrate eggs when placed in a 1 : 500 000 dilution of JG[2] for three to five minutes showed "marked differential delay or even complete inhibition of cleavage" (1).

Thus JG-B is toxic to protoplasm in varying degrees and the toxicity of this dye may well be due to its reaction with protein and to its inactivation of essential enzymes.

[1] The author did not specify which of the various types of Janus Green was used.
[2] The exact structure of the dye was not given, but it is believed to have been Janus Green B.

PROPOSED MECHANISM FOR JANUS GREEN B STAINING

Although JG-B is adsorbed on proteins, and although this dye is probably concentrated from solution by cells, there is no proof that JG-B is adsorbed to a greater extent on the proteins of the mitochondria than it is on the other proteins of the cell. The selective staining of mitochondria appears to be dependent upon a number of factors. The cell must be viable and capable of reducing JG-B. JG-B is reduced by the enzyme systems of the cell to a leuco derivative. Dead cells do not stain selectively because they are unable to reduce the dye. Supravital staining is dependent upon a delicate balance between the concentration of JG-B used, the reducing capacities of the various portions of the cell, and the factors which reoxidize the leuco JG-B formed. Presumably all portions of the cell are capable of carrying out the reduction; however, this reduction appears to take place more rapidly in the non-mitochondrial portions of the cell. Partial anaerobic conditions favor the differential staining of mitochondria presumably because it decreases the rate of oxidation of leuco JG-B. Complete anaerobic conditions abolish the mitochondrial stain because the JG-B in the mitochondria is also reduced to the leucobase. With low concentrations of JG-B, particularly when the dye is applied to the cell under partial anaerobic conditions, the amount of unreduced JG-B in the non-mitochondrial portions of the cell may never reach a concentration sufficient for visual detection and hence only the mitochondria appear to take up the dye selectively. With higher concentrations, all portions of the cell may be stained initially but the mitochondria become selectively stained as the dye is reduced more rapidly in the non-mitochondrial portions of the cell. With very high concentrations, the cytoplasm and the nucleus are stained, presumably because the reducing capacity of the cell is exceeded.

There appears to be an enzyme system within mitochondria which prevents the reduction of JG-B at this site. This enzyme system is oxygen-dependent and cyanide-sensitive. Since the cytochrome oxidase system is also oxygen-dependent, and since it is also inhibited by .001 M cyanide (36), it is probable that the cytochrome oxidase system plays a role in the supravital staining reaction. The cytochrome oxidase system of the cell seems to be concentrated within mitochondria (31, 32, 51, 55, 56), and therefore it would appear that the selective staining of mitochondria may be related to the selective localization of this enzyme system within mitochondria.

Our studies, to be reported in the following papers, on the enzymatic re-

duction of JG-B, the combination of JG-B with various cell constituents, and the reduction of JG-B by isolated cell components, lend support to this proposed mechanism of JG-B staining.

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II. REACTIONS AND PROPERTIES OF JANUS GREEN B AND ITS DERIVATIVES

S. J. COOPERSTEIN, A. LAZAROW, and J. W. PATTERSON

MATERIALS AND METHODS

The *Janus Green B* sample was purchased from the National Aniline Company. A concentrated solution was prepared by dissolving 51 mg of the dye in 50 ml of 0.1 M phosphate buffer, pH 7.4. An aliquot removed for nitrogen determination (by the micro-Kjeldahl method) contained 0.088 mg of nitrogen per ml. Assuming that all of the nitrogen was from JG-B, the maximum dye content was .534 mg/ml and the maximum purity of the sample was 52 per cent. A sample of this dye was also analyzed by the titanous chloride reduction method of Conn (1) and found to contain approximately 50 per cent JG-B. Assuming a molecular weight of 510 for JG-B, the molar concentration of this solution was calculated to be about 1.0×10^{-3} M. A dilute solution (1×10^{-5} M) was prepared by diluting with 0.1 M phosphate buffer pH 7.4. The absorption spectrum was measured in a Beckman spectrophotometer using a cuvette of 1 cm light path.

Attempts to determine the nature of the impurities in the JG-B sample indicated that it contained 8 per cent water and 11 per cent ash. Thus although the original sample contained 81 per cent organic material, only 52 per cent is accounted for by JG-B; therefore approximately 28 per cent of the sample must be non-nitrogenous organic material. An attempt was made to purify the JG-B sample by recrystallization from alcohol at $-50°$ C; however no crystals were formed. The dye was passed

through two ion-exchange resins, IRC-50 and Dowex-50. The IRC-50 resin did not adsorb the dye nor did it remove the impurities. The Dowex-50 resin adsorbed the dye; however 6N HCl was required to remove it from the resin and at this acid concentration the JG-B was destroyed.

Janus Green G was kindly supplied by Dr. E. V. Cowdry. A concentrated solution was prepared by dissolving 48.2 mg in 25 ml of 0.1 M phosphate buffer, pH 7.4. This solution contained 0.144 mg of nitrogen per ml, and assuming that all of the nitrogen was from JG-G the maximum dye content was 0.827 mg/ml. This corresponds to a purity of 43 per cent. A sample of this dye was also analyzed by the method of Conn (1) and found to be approximately 46 per cent pure. A dilute solution of JG-G was prepared by diluting the concentrated solution 1:100 with 0.1 M phosphate buffer, pH 7.4. The maximum molar concentration of dye in this dilute solution was 1.72×10^{-5} M.

Diethylsafranine. One sample of diethylsafranine was prepared by a modification of the method of Bensley [quoted by Cowdry (2)]. Sodium hydrosulfite was used as the reducing agent instead of the zinc and hydrochloric acid recommended by Bensley. The diethylsafranine was precipitated with sodium sulphate and dried. The dye was extracted with alcohol and the diethylsafranine was obtained by evaporating the alcohol solution to dryness. If all of the nitrogen in this preparation were from diethylsafranine, then the sample would be 70 per cent pure. However, since the molar extinction coefficient at 555 mμ was 20 per cent lower than the molar extinction of a sample of diethylsafranine prepared by the method described below, it is apparent that dye destruction takes place during the above preparative procedure.

The diethylsafranine used for the absorption spectra studies was prepared by reducing JG-B to leucosafranine under controlled conditions. The diethylsafranine was regenerated by diluting the solution with buffer; further purification was not attempted. 0.1 ml of 0.1 M sodium hydrosulfite was added to 5 ml of a 1×10^{-4} M solution of JG-B at pH 7.4. When the resulting leucosafranine was diluted 1:10 with phosphate buffer, the dissolved air in the buffer oxidized the excess hydrosulfite and reoxidized the leucosafranine to diethylsafranine. This solution was read in the Beckman spectrophotometer against an appropriate blank, prepared in an identical fashion except for the omission of the dye. This method of preparation minimizes the dye destruction since it was found that vigorous shaking of a leucosafranine solution, in an attempt to remove the excess hydrosulfite present, resulted in extensive destruction of the dye.

Dimethylsafranine was prepared (from JG-G) in the same manner as diethylsafranine.

Leucosafranine was prepared by adding 0.1 ml of 0.1 M sodium hydrosulfite to 3.5 ml of a 1.0×10^{-4} M JG-B solution in a Beckman spectrophotometer cuvette. When the solution became colorless (in a few seconds) a 9.0 mm quartz spacer was inserted into the cuvette. This reduces the light path of the absorbing solution to 1.0 mm. The tightly fitting quartz spacer minimizes the diffusion of air and thus the leucosafranine persists for a considerable period. This solution was then read against an appropriate blank, prepared in an identical fashion except for the omission of the dye. Although the leucosafranine solution used in the absorption spectra studies was ten times more concentrated than the other dye solutions used, the light path was only 1/10 as long.

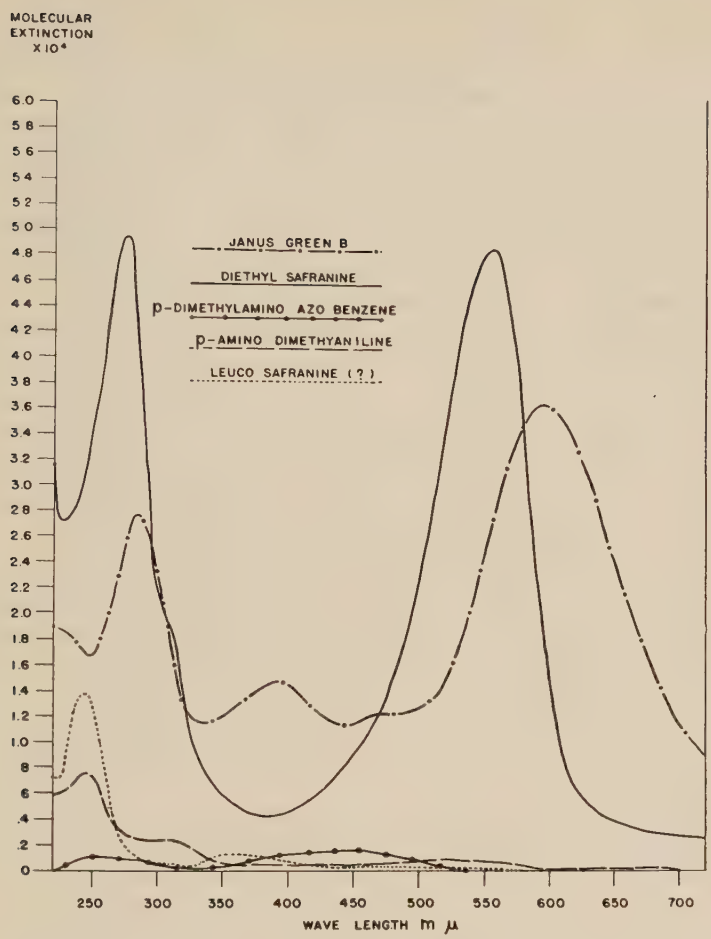

Fig. 1.

ABSORPTION SPECTRA OF JANUS GREEN AND DERIVATIVES

The absorption spectra of JG-B, diethylsafranine and leucosafranine at pH 7.4 are shown in Fig. 1. It is obvious that these spectra, especially the quantitative aspects, must be interpreted with some caution since the compounds used were not pure. The diethylsafranine curve plotted in Fig. 1 represents the sum of the extinctions of diethylsafranine plus p-aminodimethylaniline, since this latter compound is split from the JG-B during the reduction process. The spectrum for leucosafranine which is plotted in Fig. 1

is a corrected curve and presumably it also represents the spectrum of leucosafranine plus p-aminodimethylaniline. The sodium hydrosulfite solution used to reduce the JG-B has a strong ultraviolet absorption maximum at 315–320 mμ. On the other hand the oxidation products of hydrosulfite have a much lower extinction. Since hydrosulfite is used up in the preparation of leucosafranine from JG-B, the blank contains more reduced hydrosulfite than does the reduced dye solution. In fact it was found that in certain regions the extinction of the leucosafranine solution was actually less than the hydrosulfite blank. The leucosafranine curve plotted in Fig. 1 was obtained by adding 38 per cent of the extinction of the hydrosulfite blank to the extinction of the leucosafranine solution. In so doing all of the negative extinctions of the leucosafranine solution are eliminated. If more than 38 per cent of the blank value is added, a new peak appears at about 315 mμ which is the position of the absorption spectrum maximum of reduced hydrosulfite. Therefore, the curve drawn for leucosafranine is only an approximation of the absorption spectrum of this compound.

It should be noted that at pH 7.4 JG-B has a visible absorption spectrum maximum at 595 mμ (E molar = approximately 36 000) whereas diethylsafranine has its maximum absorption at 555 mμ (E molar = approximately 49 000). Leucosafranine does not have any significant absorption in the visible range. In the ultraviolet region JG-B has a maximum at 390 mμ (E molar = approximately 15 000) and at 285 mμ (E molar = approximately 28 000) whereas diethylsafranine has a maximum at 272 mμ (E molar = approximately 50 000). Leucosafranine may have a broad absorption spectrum maximum at about 360 mμ (E molar = approximately 1 200) and a second maximum at approximately 245 mμ (E molar = approximately 13 000).

Although it is possible to demonstrate that leuco JG-B is formed during the reduction of JG-B by cysteine or by zinc plus hydrochloric acid, we have not been able to stabilize this compound sufficiently to determine its absorption spectrum. If leuco JG-B-I were formed (4), the spectrum would probably approximate that of diethylsafranine. The weighting effect of the unconjugated dimethylaminobenzene might shift the maximum slightly toward the longer wave lengths and increase the absolute value of the extinction. If leuco JG-B-II were formed (4) the spectrum might be similar to that of p-dimethylaminoazobenzene (see Fig. 1)[1], whereas if leuco JG-B-III were formed (4) the spectrum would be similar to leucosafranine.

[1] The molar extinction plot of p-dimethylaminoazobenzene is only approximate. The compound has limited solubility in water and the sample did not dissolve completely.

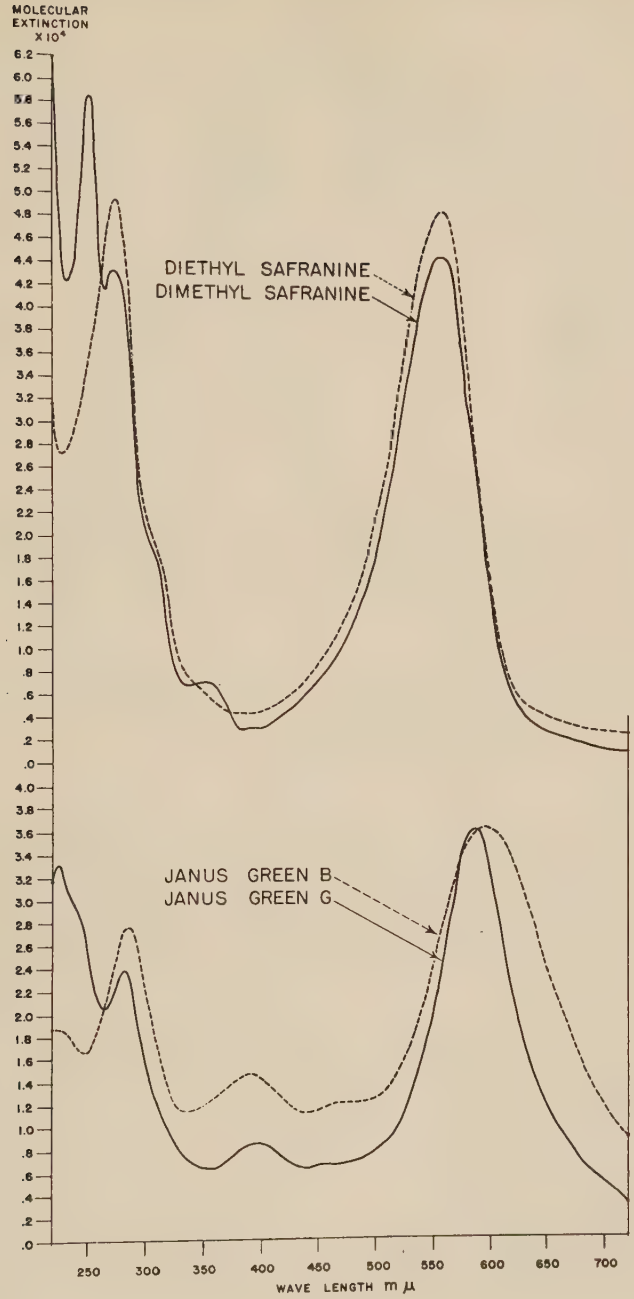
MOLECULAR EXTINCTION X 10⁵
DIETHYL SAFRANINE
DIMETHYL SAFRANINE
JANUS GREEN B
JANUS GREEN G
WAVE LENGTH mμ

Fig. 2.

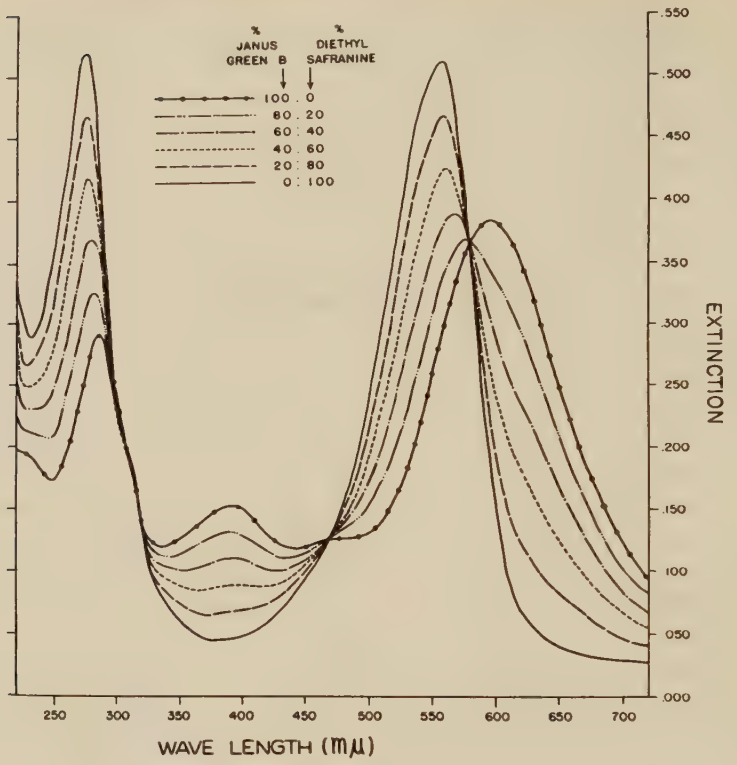

Fig. 3.

The absorption spectra of JG-G (dimethylsafranineazodimethylaniline) and dimethylsafranine are shown in Fig. 2. JG-G has absorption spectrum maxima at 587 mμ (E molar = approximately 36 000), 395 mμ (E molar = approximately 8 600) and 281 mμ (E molar = approximately 23 900). The general shape of the JG-G curve and the positions of the absorption spectrum maxima approximate that of JG-B. However there are minor differences in the positions of the maxima, and whereas the molar extinction of the visible peaks of JG-B and G are almost equal, the relative extinctions of the ultraviolet peaks differ for the two dyes. It is possible that the maximum which was observed at 225 mμ in JG-G and which was not present in the JG-B represents an impurity.

The visible peak of dimethylsafranine approximates that of diethylsafranine. It is likewise possible that the peak observed at 250 mμ in the dimethylsafranine curve is due to an impurity for this maximum is not present in diethylsafranine.

TABLE I

Calculated Positions of Absorption Spectrum Maxima of Mixtures of Janus Green B and Diethylsafranine.

Percentage Janus Green B	Percentage Diethyl-Safranine	Position of Absorption Spectrum Maximum	
		Visible	Ultraviolet
100	0	595	285
80	20	575	280
60	40	568	278
40	60	562	275
20	80	558	274
0	100	555	273

Absorption spectra of mixtures of Janus Green B and diethylsafranine. The calculated absorption spectra of mixtures of JG-B and diethylsafranine are shown in Fig. 3. It should be noted that the curves have isobestic points at 580, 468, and 318 mμ. Since the molar extinctions of JG-B and diethylsafranine are the same at these points, these wave lengths may be used to calculate the total amount of dye (JG-B plus diethylsafranine). At other wave lengths, the extinctions of the mixtures lie between the two mother curves. When mixtures of JG-B and diethylsafranine are analyzed in the spectrophotometer the amount of each component may be determined by measuring the extinction at two different wave lengths and using simultaneous equations. The relative amounts of each component can also be approximated by measuring the position of the absorption spectrum maximum (see Table I) or by measuring the ratio of the extinctions at two different wave lengths (see Fig. 4). It will be noted (see Table I) that there is a progressive shift in the position of the absorption spectrum maximum as the proportion of diethylsafranine increases.

Effect of pH on the absorption spectra of Janus Green B and diethylsafranine. The effect of pH on the absorption spectrum of JG-B is shown in Fig. 5. In the pH range of 4.6 to 8.8 the solutions were buffered with phosphate buffer. The final buffer concentration was .166 M; the JG-B concentration was 2×10^{-5} M. The extreme pH values were obtained by diluting the dye with an equal volume of 0.1 N HCl or 0.1 N NaOH. In all cases the spectra were run against an appropriate blank and the pH value of the final mixture was determined. At strongly acid pH values the visible absorption spectrum maximum of JG-B shifts from 595 to 618 mμ and there is a marked increase

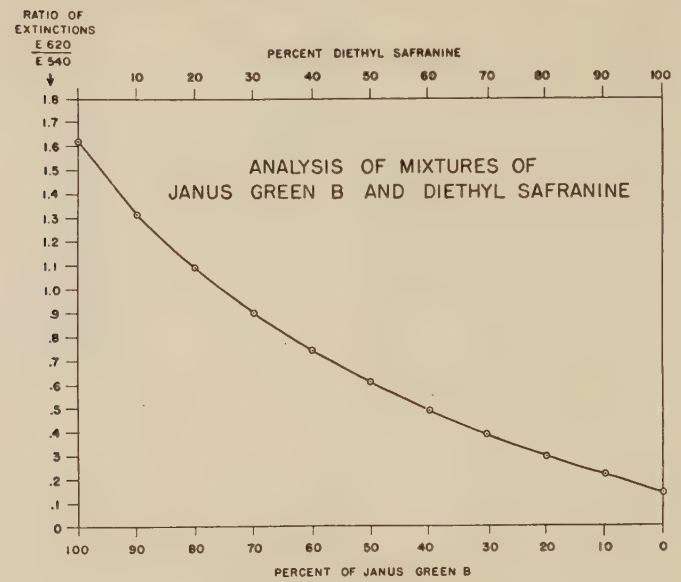
RATIO OF EXTINCTIONS
E 620 / E 540
PERCENT DIETHYL SAFRANINE
10 20 30 40 50 60 70 80 90 100
1.8 1.7 1.6 1.5 1.4 1.3 1.2 1.1 1.0 .9 .8 .7 .6 .5 .4 .3 .2 .1 0
ANALYSIS OF MIXTURES OF
JANUS GREEN B AND DIETHYL SAFRANINE
100 90 80 70 60 50 40 30 20 10 0
PERCENT OF JANUS GREEN B

Fig. 4.

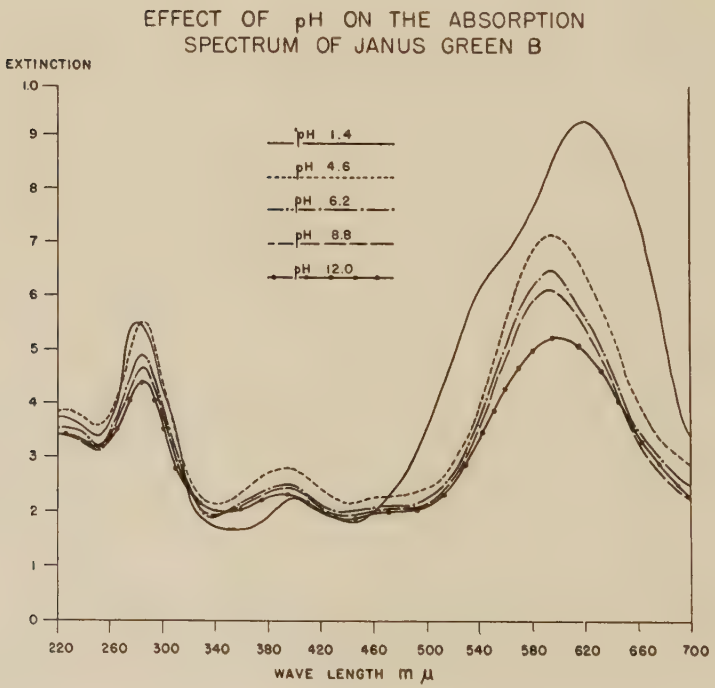
EFFECT OF pH ON THE ABSORPTION
SPECTRUM OF JANUS GREEN B
EXTINCTION
1.0 9 8 7 6 5 4 3 2 1 0
pH 1.4
pH 4.6
pH 6.2
pH 8.8
pH 12.0
220 260 300 340 380 420 460 500 540 580 620 660 700
WAVE LENGTH m μ

Fig. 5.

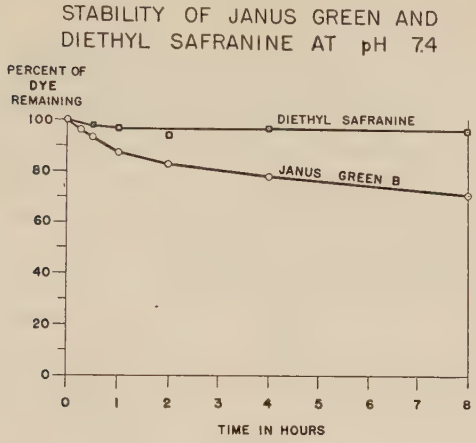

Fig. 6.

in the extinctions. An inflection appears at about 550 mμ. Visually this shift in position of the absorption spectrum maximum is associated with a color change from blue (at pH 7.4) to green (at pH 1.4). At strongly alkaline pH values, there is a decrease in the extinction values.

The absorption spectrum of diethylsafranine shows little significant change in the pH range of 1.4 to 12.0. The position of the absorption spectrum maximum does not change and in the pH range between 1.4 and 7.4 the actual extinction at 555 mμ changes by only 7 per cent.

STABILITY OF JANUS GREEN B AND DIETHYLSAFRANINE

Stability of Janus Green B solutions. Janus Green B was dissolved in 0.1 M phosphate buffer at pH 7.4 at a concentration of 1 mg per ml and placed in a Beckman spectrophotometer cell. The extinctions were read at intervals for eight hours, and the results plotted in Fig. 6. There was a 28 per cent decrease in the extinction of the JG-B solutions over the eight hour period. Some of the JG-B is adsorbed on the surface of the Beckman cuvette. At strongly alkaline pH values, solutions of JG-B are destroyed more rapidly. Aeration further accelerates the destruction; when air is bubbled through a JG-B solution at pH 12 for 15 minutes, 63 per cent of the dye is destroyed.

Stability of diethylsafranine solutions. When a diethylsafranine solution was prepared by hydrosulfite reduction and placed in a Beckman cell, there was little change in the extinction over an eight hour period (see Fig.

6). Solutions of diethylsafranine are more unstable at acid pH values and aeration further increases the destruction. Bubbling air through the solution for 15 minutes at pH 7.4 produced only 4 per cent destruction whereas at a pH of 1.5 58 per cent of the diethylsafranine was destroyed.

Destruction of diethylsafranine was also observed during the preparation of this dye. In the early experiments the JG-B was reduced to leucosafranine by sodium hydrosulfite and the diethylsafranine was regenerated by shaking the solution (to remove the excess hydrosulfite). The recovery of diethylsafranine varied from day to day and in some cases as much as 36 per cent of the dye was destroyed. It was later found that the destruction of diethylsafranine increased with decreasing concentrations of the dye and with increased periods of shaking. To minimize this destruction in subsequent preparations the JG-B was reduced in fairly concentrated solution with a minimum amount of hydrosulfite; the excess hydrosulfite was destroyed by diluting the solution with a large volume of buffer. Apparently there is sufficient dissolved oxygen in the buffer solution to regenerate the diethylsafranine.

REDUCTION OF JANUS GREEN

Reduction of Janus Green by cysteine and glutathione. Janus Green B can be reduced to leuco JG-B by cysteine. When a mixture of 0.05 M cysteine and 2.5×10^{-5} M JG-B are placed in a test tube, progressive reduction of the dye takes place, the reduction proceeding more rapidly in the lower portion of the test tube. At a certain critical interval during the reduction process (when the solution has a violet-red color) shaking the tube reoxidizes the leuco JG-B formed and restores the blue color. Although there is little doubt that leuco JG-B is present under these conditions, it has not been possible to standardize the reducing conditions sufficiently to characterize the absorption spectrum of this leuco compound.

Quantitative data on the rate of the reduction of JG-B by cysteine and glutathione are shown in Table II. Solutions of cysteine (or glutathione) were neutralized to pH 7.4 and mixed with JG-B and buffer.[1] The extinctions were determined in a Beckman spectrophotometer at 468, 540, and 620 mμ. Since 468 mμ is an isobestic point for JG-B and diethylsafranine, the decrease in extinction at this wave length is a measure of the amount of leucosafranine formed (plus the amount of dye that is destroyed). However,

[1] The solutions used in these experiments were partially freed of oxygen by boiling or by bubbling helium through them. However no other precautions were taken to maintain anaerobic conditions.

TABLE II

Chemical Reduction of Janus Green B and G.

The final concentration of the reactants were 1×10^{-5} M Janus Green B, 1×10^{-5} M Janus Green G, .05 M cysteine, .05 M glutathione, and .05 M phosphate buffer, pH 7.4. Each figure represents the average of 3 experiments.

	Time for 25 per cent reduction (minutes)	
	Janus Green B	Janus Green G
Cysteine	22[1]	>120[2]
Glutathione	86	–

since a negligible amount of leucosafranine was formed under the conditions of these experiments, the relative amounts of JG-B and diethylsafranine present were determined simply by calculating the ratio of the extinctions at 620 and 540 mμ and using Fig. 4. At 620 mμ the JG-B absorbs more than does diethylsafranine; at 540 mμ the reverse is true.

The time required for reducing 25 per cent of the JG-B originally present is given in Table II. It was found that equivalent concentrations of cysteine reduced JG-B more rapidly than did glutathione. In contrast to the findings with JG-B, Janus Green G was not appreciably reduced by cysteine.

Polarographic reduction of Janus Green B and G was carried out using the Sargent Recording Polarograph. The reduction of the dye at a dropping mercury electrode is measured by determining the flow of current as the potential is varied; the current-voltage plot is recorded automatically by the instrument. When the applied voltage reaches the value at which the dye is reduced, there is a sudden increase in the flow of current; this results in a step in the current-voltage graph. The height of the step is a function of the concentration of the dye and its mobility. The half-wave potential, i.e., the voltage at which the height of the step has reached one-half of its maximum height, is a measure of the oxidation-reduction potential of the compound.

When JG-B was reduced at three different pH values, two steps were observed. The values for the half-wave potentials of these steps are recorded in Table III. The first reduction step, which occurs at a potential of $-.001$ to $-.013$ volts, is irreversible as indicated by the analysis method of Kolt-

[1] Calculated from the end of a twenty minute lag period.

[2] In one experiment there was no evidence of reduction after 2 hours. In the other two experiments there was 7–12 per cent reduction at the end of two hours.

TABLE III

Polarographic Reduction of Janus Green.[1]

	pH[2] 6.45	pH[2] 7.48	pH[3] 8.49
Janus Green B.			
Step 1: Janus green B → diethylsafranine	−.013	−.001	−.010
Step 2: Diethylsafranine ⇄ leuco diethylsafranine	−.188	−.188	−.217
Step 2: *Janus Green G* →?		−.214	

hoff and Lingane (3).[4] This reduction step probably represents the splitting of the azo bond with the formation of diethylsafranine and p-aminodimethylaniline. The second reduction step, which occurs at a potential of −.188 to −.217 volts, is probably reversible.[4] It presumably represents the conversion of diethylsafranine to leucosafranine.

We have been unable to detect a polarographic oxidation-reduction step which would correspond to the reversible reaction JG-B ⇄ leuco-JG-B. It seems reasonable, therefore, to conclude that this step was either too small to be detected or that this reaction occurs at a potential so close to the potential at which the azo bond splits that the two steps overlap. Since the height of the first reduction step (splitting of the azo bond) was much lower than the height of the second reduction step (reduction of the azine ring) it is probable that the height of a JG-B ⇄ leuco-JG-B step would be much smaller than the first step and therefore more likely to escape detection.

The polarographic reduction of JG-G at pH 7.48 revealed only one step; the half-wave potential for this reduction corresponds to the second step observed in the reduction of JG-B. (See Table III.) The oxidation-reduction potential of JG-G calculated on the basis of the equation $E_{1/2} = Eo - .0591$ pH (3) was found to be −.258 volts.[5] This agrees moderately well with the value of −.285 volts previously obtained (5) by means of an electrometric titration with $TiCl_2$ at pH 7.4.

The absence of an oxidation-reduction step at about — .001 volts differen-

[1] Kindly carried out by Dr. Alan Powell of Case Institute of Technology.

[2] 0.1 M phosphate buffer.

[3] 0.1 M borate buffer.

[4] According to this method one plots the potential at various points in the step against the logarithm of $(I/I_d - I)$ where I is the current flowing at the corresponding potential, and I_d is the current flowing at the half-wave potential. If the reduction process is reversible, the resulting graph is a straight line and the intercept of the plot is equal to the half-wave potential.

[5] The use of this equation involves several assumptions which make the oxidation-reduction potential value so obtained subject to some error.

tiates the reduction of JG-G from that of JG-B. It is possible that this first step is also present in JG-G but that it is simply too low to be observed. This is supported by the fact that the height of the second step in JG-G was considerably less than the corresponding step of JG-B. On the other hand the failure to find the first step in JG-G might also be due to an inherent difference in the mechanism by which these two dyes are reduced.

DISCUSSION

On comparing the chemical structures of JG-B and JG-G one would hardly predict that the substitution of two methyl groups for the two ethyl groups would make any appreciable difference in chemical reactivities of these compounds. Nevertheless in contrast to the JG-B, JG-G was not appreciably reduced by .05 M cysteine and it did not have a polarographic reduction step at the half-wave potential at which the azo bond of JG-B was split. These results would seem to suggest that the azo bond of JG-G may be reduced with greater difficulty than the corresponding linkage in JG-B. However, strong reducing agents such as hydrosulfite or zinc and hydrochloric acid will rupture the azo bond of both compounds and produce the corresponding safranine derivatives.

SUMMARY

1. The absorption spectra of JG-B and its reduction products are reported. JG-B has absorption spectrum maxima at 595, 390, and 285 mμ. Diethylsafranine has absorption spectrum maxima at 555 and 272 mμ. Leucosafranine has no maximum in the visible range.

2. Janus Green G (dimethylsafranineazodimethylaniline) has absorption spectrum maxima at 587, 395, and 281 mμ. Dimethylsafranine has absorption spectrum maxima at 555 and 275 mμ.

3. Two methods are presented for determining the relative amounts of JG and the corresponding safranine derivative in mixtures of the two dyes.

4. The effect of pH on the stability of JG-B and its derivatives were studied.

5. Whereas JG-B was readily reduced by cysteine and glutathione, JG-G was not significantly reduced by cysteine.

6. The polarographic reduction of JG-B and G were studied. At pH 7.48 JG-B showed two reduction steps, one at a potential of $-.001$ volts, the other at $-.188$ volts. JG-G showed only one reduction step at pH 7.48 ($E = -.214$ volts).

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III. REDUCTION OF JANUS GREEN B BY ISOLATED ENZYME SYSTEMS

S. J. COOPERSTEIN and A. LAZAROW

In this paper we have attempted to delimit the enzyme systems which are capable of reducing JG-B (to leuco JG-B, diethylsafranine, and leucosafranine) and to determine whether the components of the cytochrome system are capable of reoxidizing leuco JG-B and leucosafranine. Earlier work had suggested that a wide variety of biological agents are capable of reducing JG-B; indeed the ability of these systems to reduce JG[1] has been used as a tool for differentiating bacteria (3, 4, 8, 25, 40), as a test for the quality of milk (17), as a means of determining physiological gradients (13, 14, 15, 16), and as a method for studying the oxidation-reduction potential of various tissues and organisms (1, 2, 38). It has been suggested (7) that this reduction process is enzymatic in nature.

In 1933 Banga *et al.* (5) reported that the lactic dehydrogenase system could reduce JG-B to diethylsafranine and to leucosafranine. They used an enzyme system consisting of washed heart muscle fortified with lactic acid and coenzyme, and found that this crude preparation reduced JG-B to diethylsafranine and partially decolorized diethylsafranine under anaerobic conditions. The first reduction step was found to be irreversible whereas the second step was reversible. The addition of pyruvic acid inhibited the decolorization, whereas if the pyruvic acid was added after the leucosafranine was formed the leucobase was partially reoxidized to diethylsafranine. Apparently an equilibrium state was reached.

[1] The authors did not specify which of the various types of Janus Green was used.

Diethylsafranine is also reported to be partially reduced by a beta-hydroxybutyric acid enzyme system (5) and by the formic dehydrogenase and lactic dehydrogenase systems of bacteria (10).

In contrast to the studies reported above, Green and Brosteaux have stated that Janus Green[1] is reduced very slowly by the lactic dehydrogenase system (26). It was important, therefore to reinvestigate the enzymatic reduction of JG-B, utilizing more purified enzyme systems, and to delimit as far as possible the component reactions involved.

EXPERIMENTAL

Glucose dehydrogenase was prepared from lamb liver by the method of Brunelli and Wainio (12), lyophilized, and dissolved in 0.1 M phosphate buffer, pH 7.4 just prior to use.

Diphosphopyridinenucleotide (DPN) was obtained from Schwartz Laboratories, Inc. In the initial studies the preparation used was approximately 60 per cent pure and in the later studies it was approximately 85 per cent pure.

The *flavoprotein* used was prepared from beef heart muscle in accordance with the method of Straub (49) as modified by Eichel and Wainio (23).[2]

Lactic dehydrogenase was prepared by the method of Green *et al.* (27) from rabbit skeletal muscle, lyophilized, and dissolved in 0.1 M phosphate buffer, pH 7.4, just prior to use.

The insoluble *cytochrome oxidase* preparation was made from pig heart according to the directions of Keilin and Hartree (33) as modified by Wainio *et al.* (53).

Cytochrome c, purchased from Wyeth, Inc., was over 90 per cent pure, based on the extinction data of Horecker and Heppel (30).

Reduced diphosphopyridinenucleotide $DPNH_2$ was prepared by mixing 18 mg of DPN, 9 mg of glucose dehydrogenase, and 375 mg of glucose in a final volume of 9 ml of 0.03 M phosphate buffer, pH 7.4. At the end of a 15 to 30 minute incubation period at room temperature, approximately 85–90 per cent of the DPN was reduced, as measured spectrophotometrically by the extinction at 340 mμ. The solution was put in a tube (closed by means of a screw cap to avoid evaporation) and placed in boiling water for five minutes. The denatured protein was removed by centrifugation or filtration.

Janus Green B, purchased from the National Aniline Company, was approximately 50 per cent pure. *Janus Green G*, kindly supplied by Dr. E. V. Cowdry, was also approximately 50 per cent pure. The purity was considered in calculation of the molar concentrations.

Diethylsafranine was prepared by the zinc-hydrochloric acid reduction method of Bensley [quoted by Cowdry (21)], except that a more dilute solution of JG-B (4×10^{-4} M) was used. The final dry powder was dissolved in a minimum amount of wa-

[1] The authors did not specify which of the various typus of Janus Green was used.

[2] Several preparations of this material were generously supplied by Dr. Walter W. Wainio of Rutgers University.

ter. The concentration of this solution was determined by measuring its extinction at 555 mμ.

Preparation of leuco Janus Green B and leucosafranine by palladium reduction. These experiments were carried out using the anaerobic spectrophotometric apparatus designed by Singer and Kearney (48).[1] One milligram of palladium black and two ml of an 8.0×10^{-5} M solution of JG-B were placed on the sintered glass funnel. The solution to which the dye was to be added was placed in the main body of the apparatus. A stream of hydrogen was passed through the main body of the apparatus and up through the sintered funnel; when the first detectable change in color of the dye was noted (from blue to violet) the direction of the stream of hydrogen was reversed. This forced the dye into the main body where it was mixed with the solution under anaerobic conditions. Immediately thereafter all outlets of the cell were closed and the spectrum taken.

Experiments in the Warburg respirometer. Most of the studies on the reduction of the dye by various enzyme systems were carried out at 37° in standard double sidearm Warburg vessels. The DPN was placed in one sidearm, the dye in the second sidearm, and all the remaining components of the enzyme system were placed in the main body. In all cases the total volume of the reaction mixture was 4 ml. After a ten minute equilibration period, the reaction was started by tipping the two components from the sidearms into the main body of the vessel. Unless otherwise stated the vessels were shaken at the approximate rate of 100 times per minute. When the mixtures were incubated overnight, the vessels were not shaken. In some experiments the vessel contents were equilibrated with various gas mixtures during the initial ten minute period. In the anaerobic experiments, the nitrogen used was purified by passing it through a series of electrically heated copper screens to remove residual oxygen. Where gas mixtures of varying oxygen content were used, the oxygen was determined polarographically by the method of Baumberger (6).

In most experiments the reduction was followed by noting the change in color of the dye. In several experiments the change in the absorption spectrum was measured in the Beckman spectrophotometer. Since the position of the maxima in mixtures of JG-B and diethylsafranine varies with the ratio of these two dyes (19) the degree of reduction can be estimated from the position of the absorption spectrum maximum. In some instances where there was heavy precipitation, the solution was centrifuged before the spectrum was taken.

RESULTS

Anaerobic reduction of Janus Green B by the glucose dehydrogenase system. Janus Green B was reduced by the complete enzyme system within four hours (see Table I). Each of the components alone had no significant reducing capacity and the reduction of the JG-B was much slower in the absence of added flavoprotein. In these experiments a heavy blue precipitate began to form within approximately 15 minutes after the addition of the dye

[1] Kindly loaned to us by Drs. Singer and Kearney.

TABLE I

Anaerobic Reduction of Janus Green B by the Glucose Dehydrogenase System.

The complete system consisted of 0.25 M glucose, 4 mg of glucose dehydrogenase, 0.5 mg of flavoprotein, 1 mg of DPN, and 0.05 M phosphate buffer, pH 7.4. All vessels contained 2.5×10^{-5} M Janus Green B.

	Time (hours)					
	1		4		24	
	Reduction Observed Visually	Position of Absorption Maximum (mμ)	Reduction Observed Visually	Position of Absorption Maximum (mμ)	Reduction Observed Visually	Position of Absorption Maximum (mμ)
Complete System	0	585	+++	560	++++	–
Complete System without Flavoprotein	0	–	0	590	+++	555
Glucose alone.....	0	–	0	–	0	590
Glucose Dehydrogenase alone	0	–	0	–	0	590
DPN alone.....	0	–	0	–	0	590
Flavoprotein alone	0	–	0	–	0	590
None	0	–	0	–	0	590

Significance of symbols: 0=no reduction; + =reduction to violet; + + =reduction to red-purple; + + + =reduction to red; + + + + =reduction to pink.

to the enzyme system. The degree of precipitation appeared to be roughly proportional to the amount of protein present.[1] It was found that the precipitated dye was invariably reduced more slowly than was the dye in solution. For example, at the end of 24 hours the solution in the vessel containing the complete system was light pink while the precipitate was violet. When air was admitted to the vessel, the solution turned red (absorption maximum at 555 mμ) while the precipitate became blue. These results suggest that the JG-B in solution had been reduced to leucosafranine whereas the dye adsorbed on the protein was reduced only to leuco JG-B. Similar results were also obtained using the other enzyme systems, and all data in the tables refer to the dye in solution.

[1] Further studies on the adsorption and precipitation of Janus Green B by proteins will be published in a separate communication.

TABLE II

Anaerobic Reduction of Janus Green B by the Lactic Dehydrogenase System.

The complete system consisted of 0.125 M sodium lactate; 10 mg of DPN, 0.5 mg of flavoprotein, 0.025 M semicarbizide, 20 mg of lactic dehydrogenase, and 0.025 M phosphate buffer, pH 7.4. All vessels contained 2.5×10^{-5} M Janus Green B.

	Time (minutes)				
	5	45		180	
	Reduction Observed Visually	Reduction Observed Visually	Position of Absorption Maximum (mμ)	Reduction Observed Visually	Position of Absorption Maximum (mμ)
Complete system	++	+++	560	–	–
Sodium Lactate + Semicarbizide	0	0	–	0	590
Lactic dehydrogenase alone	0	0	–	0	590
Sodium Lactate alone	0	0	–	0	590

Significance of symbols: 0 = No reduction; + = reduction to violet; + + = reduction to red-purple; + + + = reduction to red; + + + + = reduction to pink.

Anaerobic reduction of Janus Green B by the lactic dehydrogenase system. When employing the lactic dehydrogenase system a ketone fixative is added to remove the pyruvic acid formed. Although cyanide is used ordinarily (26), we had to select some other ketone fixing agent since we planned to study the combined effect of the lactic dehydrogenase and the cytochrome *c*–cytochrome oxidase systems on the reduction of JG-B. It was found (see Table III) that 0.025 M semicarbizide hydrochloride (neutralized with sodium hydroxide) served satisfactorily to fix the pyruvate formed, and thereby facilitated the oxidation of lactic acid.

As can be seen from Table II the complete lactic dehydrogenase system rapidly reduced the dye, whereas none of the components alone showed any significant reducing power.

The rate of reduction of JG-B depends upon the amounts of added flavoprotein, DPN, sodium lactate, lactic dehydrogenase, and semicarbizide (see Table III). In the absence of added flavoprotein, DPN, or sodium lactate, there was little, if any, reduction. In the ranges tested, the rate of reduction increased with increasing amounts of each of the components.

TABLE III

Effect of Varying the Components of the Lactic Dehydrogenase System on Reduction of Janus Green B.

In addition to the components listed below, all vessels contained 2.5×10^{-5} M Janus Green B and 0.05 M phosphate buffer pH 7.4.

Component Varied	Concentration of Components					Time Required for Reduction to Red-Purple (minutes)
	Sodium Lactate (M)	DPN (mg/vessel)	Lactic Dehydrogenase (mg/vessel)	Flavoprotein (mg/vessel)	Semicarbizide (M)	
Flavoprotein	.125	2	20	1	.025	15
				0.5		25
				0.25		35
				0		[1]
DPN	.125	0.5	20	0.2	.025	45
		0.25				60
		0.10				120
		0				[2]
Sodium Lactate	.063	1	5	0.2	0.25	35
	.025					60
	.013					180
	0					[3]
Semicarbizide	.025	1	5	0.2	.025	30
					.013	90
					.007	240
					0	405
Lactic Dehydrogenase	.125	1	20	0.2	.025	40
			10			65
			5			95
			2			320
			0			[4]

It should be noted, however, that significant reduction of JG-B occurred even in the complete absence of added lactic dehydrogenase[5] (see Table III).

[1] No reduction at the end of 5 hours; reduction to slight violet at the end of 21 hours.
[2] No reduction at the end of 28 hours.
[3] No reduction at the end of 22 1/2 hours.
[4] Reduced only as far as the violet stage (in 95 minutes). No further reduction for 23 hours.
[5] It is of interest to note that this system is also capable of rapidly reducing methylene blue anaerobically in the absence of added lactic dehydrogenase.

TABLE IV

Effect of Zinc Chloride on Reduction of Janus Green B by the Lactic Dehydrogenase System.

In addition to the zinc chloride, each vessel contained .025 M sodium lactate, 1 mg of DPN, 0.2 mg of flavoprotein, 5 mg of lactic dehydrogenase, .025 M semicarbizide, 2.5×10^{-5} M Janus Green B, and 0.05 M phosphate buffer, pH 7.4.

Final Concentration of Added $ZnCl_2$ (M)	Time Required for Reduction to Red-Purple (minutes)
0	40
6.25×10^{-7}	85
2.06×10^{-6}	270
6.25×10^{-6}	390

With some flavoprotein preparations, the rate of reduction in the absence of lactic dehydrogenase was so great that it was very difficult to demonstrate a requirement for this enzyme. In view of the requirement for DPN, flavoprotein, and sodium lactate (Table III), it would seem that the reduction of the dye in the absence of lactic dehydrogenase might be due either to a non-enzymatic reduction of DPN by sodium lactate or to the presence of lactic dehydrogenase in the flavoprotein preparation.

This question was resolved by following the rate of reduction of DPN in a Beckman spectrophotometer at 340 mμ. When only sodium lactate and semicarbizide were added to the DPN there was no reduction of the DPN even after four hours at 37°. However, when flavoprotein (0.2 mg/4 cc) was also added there was an increase in the extinction value at 340 mμ. The rate of reduction of DPN, in the presence of 0.2 mg of the flavoprotein preparation, was only slightly less (14 per cent) than that obtained when 2.5 mg of the lactic dehydrogenase preparation were added. Thus it would appear that the flavoprotein preparations contained small amounts of a highly active lactic dehydrogenase.

During the course of this work on the enzymatic reduction of JG-B it was found that longer times or much larger amounts of the enzymes were required for the reduction of some samples of JG-B. Since JG-B preparations contain zinc (24), we studied the effect of added zinc on the reduction of JG-B by the lactic dehydrogenase enzyme system (Table IV). Zinc markedly inhibited the reduction of JG-B by the lactic dehydrogenase system and it thus seems probable that the differences observed with different JG-B samples may be due to their varying zinc content.

TABLE V

Anaerobic Reduction of Janus Green B by $DPNH_2$ and Flavoprotein.

The quantities of the components used were 2 mg of $DPNH_2$ and 0.5 mg of flavoprotein. All vessels contained $2.5 \times^{-5}$ M Janus Green B and 0.07 M phosphate buffer, pH 7.4. The "blank" was prepared by mixing glucose and glucose dehydrogenase in the same manner as for the preparation of $DPNH_2$ except that water was substituted for the DPN.

Additions	Time of Incubation (minutes)	Reduction Observed Visually
$DPNH_2$ + Flavoprotein	3	+
	15	++
	60	+++
"Blank" + Flavoprotein	45	0
$DPNH_2$ alone	180	0
Flavoprotein alone	60	0

Significance of symbols: 0=no reduction; + =reduction to violet; + + =reduction to red-purple; + + + =reduction to red; + + + + =reduction to pink.

The subsequent experiments reported in this paper using the lactic dehydrogenase system were carried out with a JG-B sample that required somewhat larger amounts of the enzyme system than previously used.

Anaerobic reduction of Janus Green B by reduced DPN ($DPNH_2$). The results of earlier experiments clearly indicated that flavoprotein was required for the reduction of JG-B. Although slow reduction of JG-B was observed in the absence of added flavoprotein, this was believed to be due to the presence of small amounts of flavoprotein in the dehydrogenase preparations. Therefore substrate amounts of $DPNH_2$ were prepared and added to JG-B under anaerobic conditions. The results shown in Table V clearly demonstrate that substrate amounts of $DPNH_2$ will not reduce JG-B. However, when catalytic amounts of flavoprotein are added, the JG-B is rapidly reduced. It therefore seems reasonable to conclude that reduced flavoprotein is the immediate reactant which reduces the JG-B, and that, as is well known (20, 50), the flavoprotein is in turn reduced by $DPNH_2$.

Effect of oxygen tension on the reduction of Janus Green B. Early experiments indicated that whereas both the glucose dehydrogenase system and the lactic dehydrogenase system were capable of reducing JG-B in the presence of air this reduction was very slow, presumably because of the rapid autoxidation of any leuco JG-B formed. The experiments shown in Table VI were carried out with gas mixtures of varying oxygen contents.

Table VI

Effect of Oxygen Tension on Reduction of Janus Green B by the Lactic Dehydrogenase System.

Each vessel contained 0.125 M sodium lactate, 10 mg of DPN, 2 mg of flavoprotein, 20 mg of lactic dehydrogenase, 0.025 M semicarbizide, 2.5×10^{-5} M Janus Green B, and 0.05 M phosphate buffer, pH 7.4.

Gas Phase	Time Required for Reduction to Red-purple (minutes)
Air	235
10 per cent Oxygen — 90 per cent Nitrogen	220
5 per cent Oxygen — 95 per cent Nitrogen	30
Nitrogen	10

When the oxygen content was decreased to 5 per cent the rate of reduction was markedly increased. With an oxygen content of 10 per cent the rate of reduction of the dye by the lactic dehydrogenase system was the same as when the gas phase was air.

Anaerobic reduction of diethylsafranine by the lactic dehydrogenase system. Diethylsafranine (2.5×10^{-5} M) was reduced anaerobically by the lactic dehydrogenase system (0.125 M sodium lactate, 5 mg of DPN, 1 mg of flavoprotein, 20 mg of lactic dehydrogenase, 0.025 M semicarbizide, and 0.05 M phosphate buffer, pH 7.4). With the complete system, there was a visible decrease in the color of the solution within one hour. When the flavoprotein was omitted, the diethylsafranine was not reduced. At the end of 23 hours, no reduction was apparent in the absence of flavoprotein, whereas a large fraction of the diethylsafranine was reduced in the vessel which contained the complete system. When both vessels were opened and shaken with air, the contents of the vessel containing the complete system turned red, while there was no change in color in the vessel without flavoprotein. Thus diethylsafranine can be reduced to the leuco form by the lactic dehydrogenase system; reduced flavoprotein is the immediate reactant which carries out this reduction.

Anaerobic reduction of Janus Green G by the lactic dehydrogenase system. The reduction of JG-G by the lactic dehydrogenase system was studied under the same conditions used for JG-B. It was found (Table VII) that JG-G was reduced slightly, if at all. When twice as much DPN and four times as much flavoprotein were added, the JG-G was reduced to a violet color in

TABLE VII

Reduction of Janus Green G by the Lactic Dehydrogenase System.

In addition to the dye each vessel contained 0.125 M sodium lactate, 5 mg of DPN, 1 mg of flavoprotein, 20 mg of lactic dehydrogenase, 0.025 M semicarbizide and 0.05 M phosphate buffer pH 7.4. Each dye was used in a concentration of 1.25×10^{-5} M; in the vessel containing both dyes the total dye concentration was 2.5×10^{-5} M.

Dye	Time of Incubation (minutes)	Reduction Observed Visually
Janus Green B	20	++
Janus Green G	330	Doubtful reduction
Janus Green B + Janus Green G	20	++

Significance of symbols: 0=no reduction; + =reduction to violet; + + =reduction to red-purple; + + + =reduction to red; + + + + =reduction to pink.

160 minutes and to a red-purple color in 280 minutes. Thus although JG-G can be reduced by the lactic dehydrogenase system, higher concentrations of the reactants are required. The slow rate of reduction of JG-G is not due to an inhibition of the enzyme system by the dye or by a contaminant in the JG-G preparation, for this preparation did not inhibit the enzymatic reduction of JG-B (see Table VII).

The oxidation of leuco Janus Green B and leucosafranine by cytochrome c. Since the leuco intermediates formed during the chemical and enzymatic reduction of JG-B can be readily reoxidized by molecular oxygen, the net rate of JG-B reduction is dependent upon the rates of the forward and backward reactions. It was of interest therefore to determine whether leuco JG-B and leucosafranine can be reoxidized to JG-B and diethylsafranine respectively by the enzyme systems which are present in the cell. A 2 ml sample of a mixture of the reduction products of JG-B prepared in the anaerobic Beckman cell as previously described was added to 2 ml of oxidized cytochrome *c* (1 mg/ml in 0.33 M phosphate buffer, pH 7.4). The cytochrome *c* became partly reduced (see Fig. 1) as evidenced by the appearance of the peaks of reduced cytochrome *c* at 550 mμ and 523 mμ (31). The presence of a peak at 523 mμ instead of 520 mμ, and the asymmetry between 525 and 535 mμ are believed to be due to the presence of unreduced cytochrome *c*. (The peak of oxidized cytochrome *c* at pH 7.4 is at 530 mμ (52).) The asymmetry between 560 and 575 mμ is believed to be due to the presence of JG-B and its reduction products. The peaks of these latter compounds are presumably masked by the much denser band of cytochrome *c*.

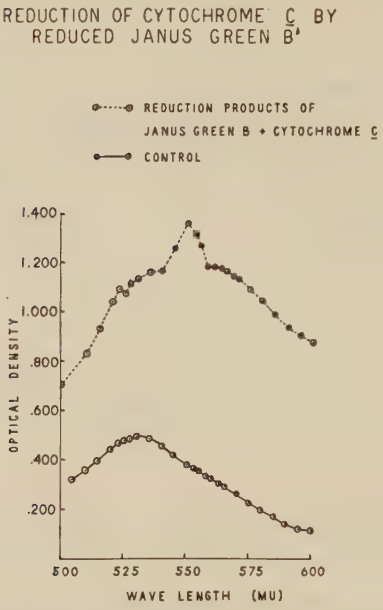

Fig. 1. The Janus Green B was reduced with palladium and hydrogen in the anaerobic Beckman cell and then added to the main body of the cuvette which contained the cytochrome *c*. The final concentration of the reactants were: 4×10^{-5} M Janus Green B, and 0.5 mg/ml cytochrome *c*.

A control experiment was carried out in the same manner except that water was substituted for the dye. The cytochrome *c* was not reduced since the peak of oxidized cytochrome *c* (at 530 mμ) was the only maximum observed (see Fig. 1).

It was also found that leucosafranine will itself reduce oxidized cytochrome *c*. When the palladium reduction of the JG-B is allowed to proceed to the colorless state, all of the JG-B is reduced to leucosafranine. When this preparation of leucosafranine is added to cytochrome *c*, the cytochrome *c* becomes reduced. However thus far we have been unable to reduce JG-B to leuco JG-B without splitting the azo bond. We have tried to limit the reduction at the leuco JG-B step by using zinc and hydrochloric acid, hydrogen in the presence of palladium black, and hydrogen in the presence of platinized asbestos. We have also tried to carry out this reduction polarographically at various pH values[1], but all attempts have been unsuccessful. Thus, although we have shown that cytochrome *c* is reduced by leucosafranine, we

[1] Kindly carried out by Dr. Alan Powell of Case Institute of Technology.

TABLE VIII

Effect of the Cytochrome *c*-Cytochrome Oxidase System on the Reduction of Janus Green B.

The aerobic reducing system consisted of 0.125 M sodium lactate, 10 mg of DPN, 5 mg of flavoprotein, 20 mg of lactic dehydrogenase, 0.025 M semicarbizide, and 0.03 M phosphate buffer pH 7.4. The anaerobic reducing system was the same except that 5 mg of DPN and 1 mg of flavoprotein were used. In the vessels containing the cytochrome system, 5 mg of cytochrome oxidase and 1 mg of cytochrome *c* were employed. All vessels contained 2.5×10^{-5} M Janus Green B.

Gas Phase	Contents of Vessel	Absorption Maxima After 30 Minutes Incubation (mμ)
5 per cent oxygen—95 per cent nitrogen	Aerobic reducing system	570[1]
5 per cent oxygen—95 per cent nitrogen	Aerobic reducing system + cytochrome system	590[2], 550[3], 520[3]
Nitrogen	Anaerobic reducing system	570[1]
Nitrogen	Anaerobic reducing system + cytochrome system	590[2], 550[3], 520[3]

have not been able to prove that leuco JG-B is also oxidized by the cytochrome system, although we believe this to be the case.

Reduction of Janus Green B by mixtures of the lactic dehydrogenase and cytochrome c-cytochrome oxidase systems. If leuco JG-B can be oxidized by cytochrome *c*, one would expect that the addition of the cytochrome *c*–cytochrome oxidase system would prevent or delay the aerobic reduction of JG-B by the lactic dehydrogenase system. When cytochrome *c* and cytochrome oxidase were added to a vessel containing the complete lactic dehydrogenase system, the reduction of JG-B was prevented. Although these results were attributed at first to the enzymatic action of the cytochrome system, this was later shown not to be the case. The addition of the colloidal cytochrome oxidase preparation resulted in an extensive precipitation and adsorption of the dye. Since it has already been noted that adsorbed JG-B is reduced more slowly than is JG-B in solution, the cytochrome oxidase preparation might have prevented reduction by simply adsorbing and precipitating the dye. This possibility was investigated by studying the reduction of JG-B by the combined systems in an atmosphere of nitrogen (see Table

[1] Peak of partially reduced Janus Green B.
[2] Peak of Janus Green B.
[3] Peaks of reduced cytochrome *c*.

VIII). The cytochrome oxidase system is not active in the absence of oxygen; nevertheless the reduction of JG-B by the lactic dehydrogenase system was inhibited even under nitrogen.

DISCUSSION

Previous workers have shown, using a washed heart muscle preparation (5) and bacterial suspensions (10), that the lactic dehydrogenase system is capable of reducing JG-B. With a partially purified enzyme system, we have demonstrated that reduced flavoprotein is the immediate reactant which reduces JG-B (and also diethylsafranine). Since reduced DPN reduces flavoprotein (20, 50), it seems reasonable to conclude that any DPN-linked dehydrogenase system would be capable of reducing JG-B. The reduction of JG-B has been demonstrated using two different DPN enzymes (glucose dehydrogenase and lactic dehydrogenase) and since this work was begun, Blakley has reported (9) that the sorbitol dehydrogenase system (in the presence of DPN) also reduces JG.[1] On the other hand, Potter *et al.* have recently reported (39) that the anaerobic reduction of JG-B by isolated mitochondria was stimulated by TPN (triphosphopyridinenucleotide) but not by DPN. It might be expected that TPN enzyme systems would be capable of reducing the dye since reduced TPN, like reduced DPN, reduces a flavoprotein (54). On the other hand the lack of stimulation by DPN, reported by Potter *et al.* is difficult to understand in view of the results reported here. We have also studied the reduction of JG-B by isolated mitochondria and other cell fractions and have found that the reduction was markedly stimulated by the addition of substrate, DPN and nicotinamide.[2] In the absence of these additions there was little, if any, reduction. It is possible that under the conditions used by Potter *et al.*, the mitochondria may have contained sufficient quantities of bound DPN (but not TPN) to saturate the system and hence they did not observe stimulation when DPN was added.

A comparison of the data on the reduction of JG-B and diethylsafranine would suggest that diethylsafranine is reduced more slowly by the lactic dehydrogenase system than is JG-B. It is possible that this slower reduction rate may be more apparent than real because it is easier to detect the color change which results from the reduction of JG-B. However, the findings of Rapkine and others (41, 42, 55) that the change in color from red to colorless occurs at a more negative potential than does the change from blue to

[1] The authors did not specify which of the various typus of Janus Green was used.
[2] These studies will be reported in a separate communication.

red suggest that the slower rate of reduction of diethylsafranine may have a chemical basis.

JG-G (the dimethylsafranine derivative) was reduced extremely slowly by the lactic dehydrogenase system. This difference in reducibility between JG-G and B may serve to explain the apparently contradictory results reported by Green and Brosteaux (26). These investigators have stated that JG was not reduced by the lactic dehydrogenase system but they did not specify the type of JG used. If these investigators had used JG-G, their negative results would be readily explained.

These observations on the relatively slow enzymatic reduction of JG-G are similar to the polarographic and the cysteine reduction studies (19). They suggest that there is an inherent difference in the chemical and enzymatic reducibility of JG-B and G. Since the rate of reduction of JG-B by the lactic dehydrogenase system is not affected by the addition of JG-G, the slow enzymatic reduction of JG-G is not due to an inhibitor in the preparation, and JG-G does not act as a competitive inhibitor toward the reduction of JG-B.

These findings are in keeping with Cowdry's studies (21) on the chemical specificity required for the JG staining reaction. JG-G, unlike the diethylsafranine derivative, does not stain mitochondria selectively and it would appear that selective staining is dependent upon the ability of the cell to reduce the dye.

It has been shown that cytochrome *c* is reduced by leucosafranine and the leucosafranine is in turn oxidized to diethylsafranine. It is presumed from the work of Lewis and Lewis (36, 37)[1] and Brenner (11) that leuco JG-B also may be reoxidized (to JG-B) by the cytochrome *c*–cytochrome oxidase system, but this has not been proven. These investigators have shown that when supravitally stained cells are treated with .001 M cyanide, the JG-B is decolorized. When the cyanide is removed by washing the JG-B is regenerated. It therefore appears that a cyanide-sensitive enzyme system is active in the supravitally stained cell and that this enzyme system acts to maintain the dye in the oxidized (blue) form. Since cytochrome oxidase is inhibited by .001 M cyanide (32), it seems likely that the cytochrome *c*–cytochrome oxidase system is the enzyme system involved. The localization of the cytochrome *c*–cytochrome oxidase system within the mitochondria (22, 28, 29, 43, 44, 45, 46, 47) could prevent the reduction of JG-B at this site in at least two ways: 1) Since most flavoproteins are oxidized by cyto-

[1] The authors did not specify which of the various types of Janus Green was used, but it was believed to have been Janus Green B.

chrome *c* (51), the presence of an active cytochrome *c*–cytochrome oxidase system in the mitochondria would keep a larger percentage of the flavoprotein in the oxidized state. Since reduced flavoprotein is the immediate reactant which reduces JG-B, the rate of decolorization of JG would be decreased. 2) The presence of the cytochrome *c*–cytochrome oxidase system could slow the net rate of JG-B reduction by reoxidizing the leuco-JG-B formed.

SUMMARY

Janus Green B is reduced to diethylsafranine and to leucosafranine by the lactic dehydrogenase and the glucose dehydrogenase enzyme systems. Reduced flavoprotein is the immediate reactant which carries out this reduction and therefore any DPN-flavoprotein enzyme system should be capable of carrying out this reduction. Cytochrome *c* can reoxidize the leucosafranine formed and it is postulated that the cytochrome *c*–cytochrome oxidase system is also capable of reoxidizing leuco JG-B. Janus Green G is not readily reduced by the lactic dehydrogenase system. These results have been discussed in relation to their significance in the JG-B staining reaction.

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A STUDY OF THE MEDULLARY CELLS OF THE HAIR[1]

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Keratins are known to be fibrous proteins of epidermal and skeletal tissues containing a characteristically high amount of sulfur. They are insoluble in the usual protein solvents but are slowly attacked by moderately strong solutions of alkali or reducing substances (15, 22, 24, 34). Medullary keratin of the hair of carnivores, rodents, and some other species shows a significant departure from usual keratins (2, 3, 5, 21, 31, 32, 33) since it contains little if any sulfur and resists the action of strong alkali and reducing substances. This atypical keratin was studied in goat hair by Lloyd and Marriott (21). It was noticed that while the cortex of the hair readily dissolved in 4 M solution of sodium hydroxide like ordinary keratins the medullary cells resisted its action and appeared unaltered under the microscope. Sulfur determinations showed that the cortex contains a relatively high amount of sulfur, 3.6 per cent, while the medullary cells contain only 0.23 per cent.

Stoves (33) studied the medullary cells of a number of animal fibers and observed that the size of the cells diminished and their surface developed a puckered appearance if they came in contact with tannic acid. Microblisters were also seen after contact with sodium oleate or saponin. These observations suggested that the surface structure of the medullary cells may contain a lipo-protein-cholesterol combination which might protect the cells from strong alkali action. Cholesterol was demonstrated by the Liebermann-Burchard reaction.

The chemistry of wool and other types of keratins has been fairly intensively studied, but apart from the above data not much is known about the nature of medullary cells or the properties of medullary keratin. The purpose of the present study was to obtain further knowledge on medullary cells of the hair. For this study the hair of the white rabbit was se-

[1] This work was supported by a grant from the American Cancer Society.

lected since in this type of hair the cortex is poorly developed and the bulk is made up of large medullary cells. The medullary cells were isolated by a modified method of Lloyd and Marriott (21). The structure, properties, and composition of the medullary cells were investigated as described here.

MATERIAL

Hair was clipped from white rabbits which were kept in a constant temperature room and fed a standard laboratory diet. The microscopic structure of the hair is shown in Fig. 1. The main part of the hair is made up of large rounded medullary cells enclosed by a thin layer of flattened cortical cells. The medullary cells are arranged in single or more often double or multiple rows. For the purpose of establishing a reference standard a total N of 14.5 per cent and a cystine content of 1.4 per cent were determined for lipoid free hair.

METHOD OF ISOLATION OF MEDULLARY CELLS

In preliminary studies the medullary cells were isolated by suspending the hair in 4 N sodium hydroxide for 24 hours at room temperature, according to the method of Lloyd and Marriott (21). The total nitrogen content of isolated medullary cells was estimated to be between 12 and 13 per cent. This relatively low nitrogen content suggested that the strong alkali treatment caused some degree of chemical degradation of the material. Therefore in succeeding experiments weaker solutions of alkali were used. It was found that the cortex readily dissolved if the hair was first treated with 0.7 N potassium hydroxide followed by 3 N potassium hydroxide. The medullary cells showed a total nitrogen content of between 14.0 and 14.5 per cent. On the basis of this observation the following method was established and used for the isolation of medullary cells.

In a single experiment usually 50 g of hair were suspended in 2 000 ml of 0.7 N potassium hydroxide and stirred for one hour. During this period of time the cuticle and cortex partially dissolved, but the hair did not fall apart. Dust particles and impurities were removed from the hair and suspended in the solution. After this the alkali was washed away by repeated suspension of the hair in distilled water. The clean material was then suspended in 1 000 ml of 3 N potassium hydroxide and stirred for one hour. The viscosity of the suspension increased as the hair disintegrated and the cuticle and cortex dissolved. After one hour's stirring the suspension was allowed to stand at room temperature for exactly 17 hours. During this period of time the cortex completely dissolved, while the medullary cells sedimented to the bottom of the beaker. The supernatant was a transparent yellow fluid, which was discarded. The suspension which contained the medullary cells was filtered successively three times through coarse glass wool and ordinary muslin to remove some undissolved particles of the hair. Microscopic examination of the filtrate showed that it contained mainly medullary cells and a small quantity of

small granules and cell fragments. The medullary cells were separated from the other particles by repeated centrifugations which were done at 1 400 G for 30 minutes. The medullary cells sedimented, while the small particles remained in the supernatant, which was discarded. The medullary cells were sedimented and resuspended successively in 1.5–0.7 N potassium hydroxide and finally three times in distilled water. The sediment of the last centrifugation was a whitish translucent greasy substance. Microscopic examination showed that it consists entirely of medullary cells. In the final step of preparation the material was rendered alkali-free by repeated washing in distilled water and by dialysis against running distilled water for 24 hours or more.

For the chemical analyses the material was dried at 110° C to constant weight and pulverized. The dry material looked yellow and was of a uniform consistency, like horn.

When medullary cells were isolated by this method from gray hair, the preparation looked black. The black color was due to the pigment of the hair, which was in the body of the medullary cells and which did not dissolve in the course of the isolation procedures.

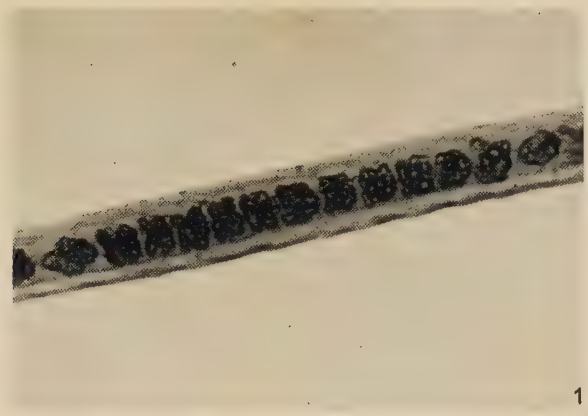
MICROSCOPIC EXAMINATION OF ISOLATED MEDULLARY CELLS

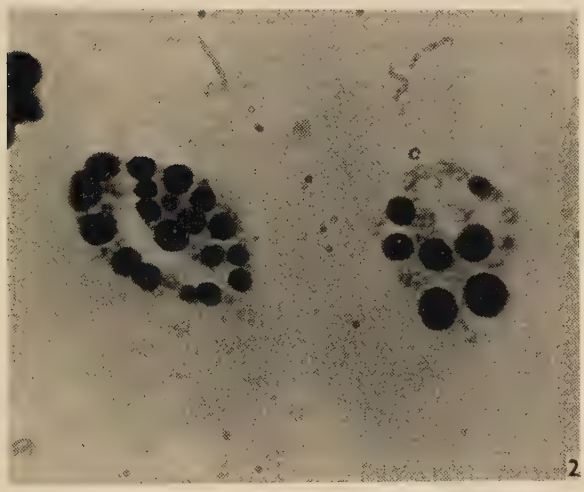
Medullary cells of rabbit hair *in situ* appear as structureless, coarse, highly refractile bodies. The isolated medullary cells appeared pale and swollen and revealed more structural details. They were discoid and rounded, varying widely in size. No nucleus or cellular membrane or continuous cytoplasm could be discerned. The cells were made up of large and small granules which were attached to one another. Fig. 2 is a photomicrograph of isolated medullary cells which were stained with safranine.

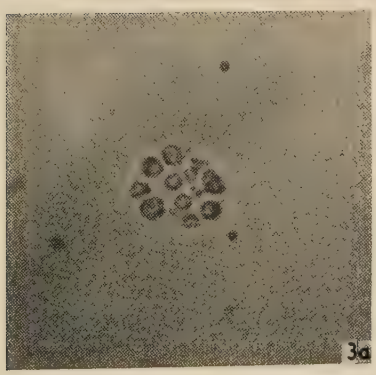
The isolated cells stained equally well with hematoxylin, eosin, methylene blue, toluidine blue, Janus Green or safranine. The Feulgen reaction was negative. Chèvremont and Frédéric's technique (12) was used to study reactive SH groups in the material. Negative results were obtained with and without pretreatment with cyanide.

The isolated medullary cells showed no sign of double refraction in the polarizing microscope. When rabbit hair was fragmented in the Waring blendor and suspended in distilled water, the separated medullary cells also appeared isotropic. The isotropic character of isolated medullary cells is not a result of the action of strong alkali but appears to be the true property of the cellular material.

In the phase-contrast microscope the granules of medullary cells are better seen; they show more contrast and appear similar to keratohyalin granules of epidermal cells.







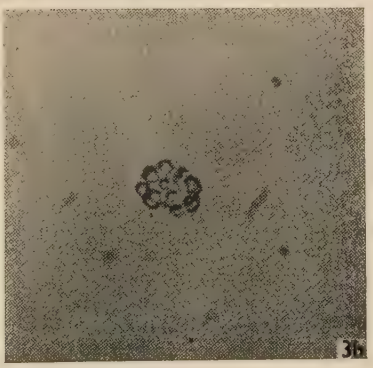


Fig. 1. Microscopic structure of white rabbit hair.
Fig. 2. Isolated medullary cells of rabbit hair stained with safranine.
Fig. 3. Microphotographs show the acid shrinkage of an isolated medullary cell. A. The cell suspended in distilled water. B. Same cell suspended in weak hydrochloric acid.

No alteration was observed in the structure of isolated medullary cells when they were suspended in salt or urea solutions, but a marked volume change was noticed in acid solutions. When a drop of aqueous suspension was examined under the microscope and 0.01 N hydrochloric acid was diffused into the cells, they suddenly shrank to a small volume (Fig. 3). The shrunken cells appeared most similar to the medullary cells of the unaltered hair (Fig. 1). The structural details of such cells were not easily detectable. When the same preparations were perfused with 0.01 N potassium hydroxide, the shrunken cells regained their volume. The acid shrinkage and alkali swelling was reversible and it could be repeated several times. The shrunken cells appeared to be about half of their original size.

QUANTITATIVE STUDIES ON THE VOLUME CHANGES OF ISOLATED MEDULLARY CELLS

Whereas hair or wool fibers swell very little over practically the whole pH range (24), medullary cells show marked volume changes on reaction with acid or base. A quantitative study of this phenomenon seemed advisable in order to obtain data on the capacity of water uptake as well as on the amphoteric nature of the material.

Into a series of special graduated centrifuge tubes (Corning No. 8360) 4 ml of a concentrated aqueous suspension of medullary cells were pipetted and the volume made up to 10 ml with glycine-hydrochloric acid-sodium hydroxide buffers, which ranged in pH from 1.4 to 12.0. The tubes were shaken for about 2 hours to reach equilibrium. All the suspensions were centrifuged for exactly 20 minutes at 1800 G under identical conditions. The volume of each of the sediments was read on the scale of the centrifuge tube and the pH of the suspensions was determined in the Beckman pH meter. The volume of the sediments was plotted against pH values. The curve obtained is shown in Fig. 4. It shows that medullary cells have about the same volume at neutrality as in alkali, while in acid different degrees of shrinkage occur. The minimal point of swelling is between pH 4.0 and pH 4.5. The marked swelling from pH 4.5 up to pH 6 is of particular interest since it shows an increase of volume around 1000 per cent. The flat part of the curve from pH 7 to 12 indicates that the material is not sensitive to the action of alkali within this pH range.

It will be shown later that protein constitutes 96 per cent of the dry medullary cells, an observation which suggests that the protein component is

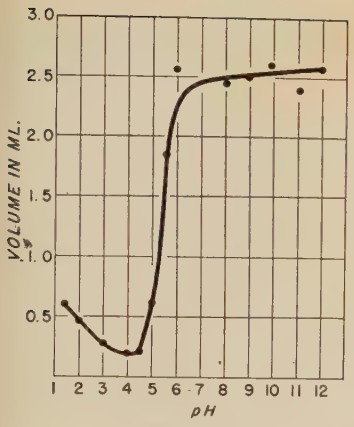


Fig. 4.

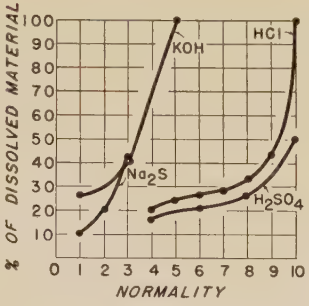


Fig. 5.

Fig. 6.

Fig. 4. Volume changes of fresh medullary cells in glycine-HCl-NaOH buffers.
Fig. 5. Dissolution of fresh medullary cells by strong acid, alkali, and reducing substances.
Fig. 6. Absorption spectrum of 0.07 per cent aqueous suspension of fresh medullary cells.

responsible for the changes in volume. It is known that the minimal point of swelling of a protein coincides with its isoelectric point. According to this, the measurements indicate that the isoelectric point of medullary keratin lies between pH 4.0 and 4.5. The isoelectric point of wool keratin is known to be at pH 4.2 (29). Therefore the isoelectric point of medullary keratin seems to be close to that of wool keratin.

For the correct interpretation of the changes in volume of medullary cells it must be remembered that the material has been treated with strong alkali and because of this some alterations might have occurred in the protein molecules to influence the water uptake. Lloyd and Marriott (22) showed that on the action of alkali nonreversible alterations take place in keratins, such as the opening of a coordinate link between the carbonyl groups of one peptide chain with the imino group of a parallel adjacent chain or the opening of the peptide links between the acid and basic R groups of adjacent chains. The binding of water molecules might occur in freed groups. In alkali-treated wool fibers a higher degree of swelling with two maxima were found at pH values of about 2 and 10. Although in the present investigation such characteristic maxima were not found, it is very probable that the enormous swelling of the medullary cells was influenced by pretreatment with alkali.

THE REACTIVITY OF MEDULLARY CELLS TOWARD USUAL KERATIN SOLVENTS, STRONG ALKALI, REDUCING SUBSTANCE, AND ACIDS

Goddard and Michaelis (15) showed that 0.5 M thioglycolic acid, sodium sulfide, and cyanide dissolve wool keratin. This effect was ascribed chiefly to the splitting of the disulfide groups which are essential for the maintenance of the structure of keratin. Medullary cells resisted the action of these solvents, which indicates that their structure is stabilized by a type or types of bond other than -S-S- bridges.

To gain some knowledge of the degree of resistance of medullary cells their dissolution was studied on the action of potassium hydroxide, sodium sulfide, hydrochloric acid, and sulfuric acid, all of which were used in solutions varying from 1 to 10 N in concentration. Four milligrams of medullary cells of known nitrogen content were suspended in 10 ml of each solution and kept at room temperature for 24 hours. The suspensions were shaken intermittently. The undissolved materials were sedimented by centrifugation and their nitrogen content was determined. The amount of the dissolved material was calculated on the basis of comparison of the original nitrogen content with that of the sedimented fraction. The percentage of dissolved materials was plotted against the normality of the solutions. The curves obtained are shown in Fig. 5. It can be seen that potassium hydroxide was the most effective solvent. The action of sodium sulfide was similar to that of potassium hydroxide. A solution of 5 N potassium hydroxide completely dissolved the medullary cells, while the same result was obtained when 10 N hydrochloric acid was used. The action of sulfuric acid was much milder; a 10 N solution dissolved only half of the amount of medullary cells dissolved by 10 N hydrochloric acid. These results show that medullary cells are much more resistant to concentrated acids than to alkali or reducing substances.

In the preceding experiments the order of dissolution of medullary cells appears to follow the order of hydrolyzing action of the chemicals. As was known (16) the most rapid hydrolysis of a protein is effected by 5 N alkali, while if hydrochloric acid is used, hydrolysis is slower and requires a higher concentration (8 N solution). Sulfuric acid is known to be the slowest-acting hydrolyzing agent.

Similar results were obtained when the above experiments were performed on both intact fresh material and dry lipoid-free material. This indicates that the high resistance to dissolution of medullary cells might

be attributed to the resistant structure of medullary keratin (5) rather than to a protective lipoid membrane which covers these cells (33).

THE COMPOSITION OF ISOLATED MEDULLARY CELLS

The chemical analyses of isolated medullary cells show that they are of a complex composition. Three main components were identified: a lipoid, a carbohydrate, and a protein component, the last being present in the largest quantity.

Lipoid component. The extractibility and solubility of the lipoid component was investigated using ethanol, chloroform, ether, and mixtures of them. Extractions were made in a Soxhlet apparatus for two days and in each case about 200 mg of substance were used. After evaporation of the solvents the amount of extracted substance was determined gravimetrically. Ethanol was found to be the most effective solvent, followed by chloroform, and then ether. The amounts of extracted substances were calculated as percentage of dry medullary cells: ethanol 3.4, chloroform 2.1 and ether 0.8. When mixtures of solvents were used, the results varied within the above ranges.

The lipoid component was found to represent a mixture of different materials, some of which were identified. The composition of ethanol-extracted lipoid was studied in detail. Cholesterol was determined by the Liebermann-Burchard method, which showed that 14.7 per cent of the lipoid component is cholesterol. On this basis the percentage of cholesterol in the isolated medullary cells is 0.50 per cent. The lipoid component contained 0.01 per cent phosphorus, which was determined by the method of Fiske and Subbarow (13). The low phosphorus value reveals that phospholipids are present only in small quantities.

In the chloroform and ether extracts only cholesterol was determined. Calculations showed that in the case of chloroform extraction 0.53 per cent of the entire medullary cell is cholesterol, which is nearly the same value obtained in the case of ethanol extraction (0.50 per cent). In the case of ether extraction a value of 0.37 per cent was obtained, which indicates that only a part of the cholesterol was extracted from the medullary cells.

Carbohydrate component. The presence of carbohydrates was first suspected when a violet coloration of medullary cells was noticed in concentrated hydrochloric acid. As is known (14), a violet color is given by tryptophane in the presence of a carbohydrate in concentrated hydrochloric acid. Since medullary cells showed this color in hydrochloric acid without

the addition of a carbohydrate, it is suspected that they contain a carbohydrate component which reacts with tryptophane of medullary keratin.

The presence of carbohydrates in isolated medullary cells was also demonstrated by a more specific reaction, the Molisch test, which gave a positive result. The amount of carbohydrates was determined by the method of Sörensen and Hangaard (30). This gave a value of 0.7 per cent. The nature of the carbohydrate component has not yet been determined.

Protein component. Ethanol-extracted medullary cells were assumed to represent the protein component, which will be referred to as medullary keratin. Total nitrogen determinations were made according to the Pregl modification of the Kjeldahl method. The average *N* value of the preparations was 14.4 per cent. The total nitrogen content of the keratins varies between 14.9 and 16.9 per cent (6). It seems that the somewhat low N value of medullary keratin is a consequence of a mild degradation of the material which occurs during the process of isolation. Negative results were obtained for phosphorus by the method of Fiske and Subbarow (13).

Medullary keratin was further characterized by cystine and aromatic amino acid determinations. Cystine was estimated according to the Block and Bolling modification of Folin and Winterstein's method (7). The value of 0.28 per cent was obtained. This result is in agreement with that found by previous workers (2, 3, 5, 21) and shows that medullary keratin contains a significantly small amount of cystine.

The presence of aromatic amino acids was shown by a spectroscopic method. The ultraviolet absorption spectrum of a 0.07 per cent aqueous suspension of medullary cells was determined in the Beckman spectrophotometer. The absorption curve obtained is presented in Figure 6. It shows a characteristic peak with its maximum at 275 mμ wave length. This maximum is characteristic for tyrosine or tryptophane which show a specific absorption around this range of ultraviolet light. Since the medullary cells scatter incident light, the absorption curve cannot be used for a quantitative study. The amount of aromatic amino acids was determined by the colorimetric method. Tyrosine was estimated by Bernhart's modification of the Millon-Weiss method (4), tryptophane by Shaw and McFarlane's modification of the Hopkins-Winkler method (28) and phenylalanine by Kuhn and Desnuelle's modification of the Kapeller-Adler method (19). The following results were obtained which are given as percentages of the medullary keratin: 2.3 tyrosine, 11.9 tryptophane and 6.4 phenylalanine.

The results show that about 20 per cent of medullary keratin is made

up of aromatic amino acids. The total amount and distribution of these amino acids, especially tryptophane, differ in hair or wool fibers. Tyrosine is known to vary between 2.4 and 5.9 per cent, tryptophane between 0.8 and 1.5 per cent, and phenylalanine between 1.5 and 4.5 per cent (6), and the total amount of aromatic amino acids varies between 4.7 and 11.9 per cent. The presence of tryptophane in large quantities appears to be a characteristic property of medullary keratin, one which distinguishes it from other keratins.

On the basis of the above chemical analyses the composition of isolated medullary cells of white rabbit hair might be summarized as follows: 95.9 per cent protein, 3.4 per cent lipoids, and 0.7 per cent carbohydrates.

DISCUSSION

Medullated hair or wool fibers represent an interesting biological association of two different types of keratin. In the cortex a usual type of cystine-rich keratin is present (2, 3, 5, 21) with a highly organized structure (23), while in the medulla there is a sulfur-poor atypical keratin with an unorganized structure. Since both structures appear to have a common cellular origin, basic differences must exist in the processes of formation of these different proteins. On the basis of our present knowledge of keratinization the following hypotheses may be made in connection with the formation of cortical and medullary keratin.

The structure and composition of cortical cells of medullated fibers have been found largely identical to that of nonmedullated hair or wool fibers (5, 23). This suggests that keratin formation in cortical cells proceeds under similar conditions. It is known that the precursor molecules of hair keratin contain SH but no SS groups (25, 34) and show a propensity to aggregate in cylindrical micelles (1). During keratinization this gel-like material becomes hardened and the micelles are linked by S-S bonds of cystine and the structures become stabilized in a highly organized and oriented form (1, 25, 34). Thus cysteine and cystine molecules are important in the formation and fixation of cortical keratin molecules.

The keratinization of medullary cells must differ in mechanism from that of cortical cells. The absence of double refraction and of a characteristic x-ray pattern of medullary cells (33) indicates the lack of an organization mechanism during keratinization. The absence of cystine might be responsible for the lack of fixation of the molecules into organized structure. Probably the loss of water, the gradual shrinkage, and the drying-

up processes are the main factors in keratinization of medullary cells. During such processes the micelles might cluster together in random fashion, forming unorganized structures.

The building up of the resistant cohesive forces in medullary cells is still an unknown mechanism, and the understanding of these forces awaits further research. Concerning the alkali-resistant nature of medullary keratin, it is interesting to recall that the diffraction pattern of porcupine-quill medullary cells shows the presence of a β-type of keratin (23). This type of medullary cell is also resistant to strong alkali. Blackburn (5) suggested that their high resistance is related to their structure. He pointed out that the amino acids of β-keratins might be predominantly those having short nonpolar side chains and he suggested that since such fully extended polypeptide chains pack more closely together than those of the folded chains of α-keratins, they are consequently less accessible and less affected by reagents such as strong sodium hydroxide.

Medullary cells originate from metabolically active epithelial cells of the hair follicle. In the course of their upward movement their nourishment becomes inadequate and the cells undergo different metabolic and morphologic changes. Finally they become transformed to shrunken masses of coarse granules. The negative Feulgen reaction of isolated medullary cells and the absence of phosphorous from medullary keratin suggests that during keratinization the nucleoproteins completely disappear from the epithelial cells. At present there is no information as to whether the nucleoproteins play a role in the synthesis of medullary keratin as they do in the synthesis of other types of proteins (8, 9, 10, 11).

In connection with the carbohydrates shown to be present in medullary cells, it might be recalled that Sylvén (35) suggested that hair develops in a matrix which is rich in polysaccharides. Glycogen has been demonstrated in large quantities in different cells of the hair follicle (18, 20, 26). In our present state of knowledge it is not possible to propose any relationship between these isolated observations.

Lipoids are known to be associated with keratins (14) and are thought to play some role in keratinization (27). Since medullary cells seem to pass through degenerative stages during their keratinization, it is possible that the lipids of medullary cells have their origin in such regressive changes. The accumulation of cytoplasmic lipids in degenerative processes or under inadequate nutritional conditions, such as in old tissue cultures, is an established phenomenon. It is clear that such speculation is based upon indirect evidence.

SUMMARY

1. Medullary cells of white rabbit hair were isolated by a modified method of Lloyd and Marriott.

2. Microscopic examination of isolated medullary cells shows that they are made up of both large and small granules. Medullary cells appear isotropic under the polarizing microscope. On the action of acid the cells show marked shrinkage and regain their volume upon alkali action.

3. The minimal point of swelling of medullary cells is between pH 4.0 and 4.5, indicating that the isoelectric point of medullary keratin is between these pH values. Between pH 4 and 6 the volume of medullary cells increases about 1 000 per cent.

4. Medullary cells did not dissolve in the usual keratin solvents, indicating that medullary keratin is an atypical keratin. They were solubilized in 5 N alkali or 10 N hydrochloric acid in the cold within 24 hours.

5. Medullary cells were found to be composed of lipoid, carbohydrate, and protein components, the last making up the largest percentage. It was found to be characteristic of medullary keratin that it contains minute quantities of cystine and a higher amount of tryptophane than common keratins.

6. A possible mechanism of keratinization of medullary cells is discussed.

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OBSERVATIONS ON THE SOCIAL BEHAVIOUR OF CELLS IN TISSUE CULTURE

I. SPEED OF MOVEMENT OF CHICK HEART FIBROBLASTS IN RELATION TO THEIR MUTUAL CONTACTS

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A tissue culture is often referred to as a colony of cells, thereby implying that a cell can in some circumstances be regarded as a social organism. Yet this idea seems to have had surprisingly little effect on methods of investigation. No systematic attempt, so far as we are aware, has been made to apply to cellular societies the analytical approach which has been so successful in the study of animal social interaction: that is, to take the cell as a unit and to investigate how its behaviour is influenced by other cells. There are, of course, many valuable incidental observations of the behaviour of individual cells as it is affected by their neighbours, notably in the papers of Willmer and his colleagues (16), of Twitty and his colleagues (11) and of Holtfreter (5). But most investigations of cell colonies have been less concerned with the individual cell than with the overall patterns of distribution taken up by whole populations of cells. In an attempt to develop an analysis of individual cell behaviour and the influence of other cells upon it, using behaviour in the narrow but commonly accepted sense of movement, we have made a series of observations and experiments on cells in tissue culture.

In the present paper we deal with chick heart fibroblasts in the highly simplified conditions of liquid medium culture, and we are concerned only with the hypothesis that the velocity of the bodily displacement of a cell tends to be affected by its contacts with other cells. This hypothesis was suggested to us in general by the well-known views of Holfreter (4) and Weiss (14, 15) as to the importance of mutual contact for cell behaviour, and specifically, by an effect which contact with axons within the explant seemed to have on the migratory activity of Schwann cells in tissue cultures of regenerating peripheral nerves (1). We wish to do no more at present than to demonstrate the occurrence of two relationships between velocity and contact: one between the velocity of a cell and the number of other cells

with which it is in contact, and the other between velocity and the act of losing or gaining contact with other cells. Within our cultures velocity has proved to be more conspicuously related in these two ways than in any other. We are not, however, concerned at present with how frequently and how strongly amongst different fibroblast cultures these relationships show themselves. We have evidence that the relationships are variable and may occasionally be absent. Our object is merely to demonstrate the existence of these relationships, not hitherto described, and we therefore confine ourselves to an intensive analysis of five cultures in which they occur.

MATERIAL AND METHOD

Explants were taken from the ventricles of chick embryos of seven to ten days incubation, and cultured on cover slips by the hanging drop method. Observations were made on the primary culture without transplantation. The medium consisted of 2 parts fowl plasma, 1 part embryo extract made originally at 50 per cent dilution from 7-day chicks, and 1 part Pannett and Compton saline. In four of the cultures analysed, in order to obtain an experimentally increased amount of cell interaction, two explants separated by 0.7–1.0 mm were placed on each cover slip. In the remaining culture a single explant was used, to show that the effects observed were not peculiar to double-explant cultures. The plasma-extract mixture containing the explants was allowed to clot, and then an area of liquid medium was prepared by cutting away the clot between the two explants or, when there was only one explant, in a region bordering it. The space left by such an excision immediately fills with fluid exudate from the surrounding clot. This method is due to Miss Honor Fell.

Our data come from filming the cells which moved into the area of liquid medium. One film was taken of each of the double-explant cultures, and two consecutively at different places in the zone of outgrowth of the single-explant culture. The filming was done for us by Mr. R. McV. Weston of Messrs. SIMPL Ltd. We used a phase contrast microscope with a 2/3″ objective, 16 mm film, and one exposure every 20 seconds. The cultures were maintained at 38° C during filming, which usually started after some 12 to 18 hours preliminary incubation and was continued for a further 5 to 12 hours. Data about the films is given in Table I.

Most of the analysis of each film was made by means of a series of enlarged prints of individual frames. The prints were taken at intervals of forty frames (one foot of film, the equivalent of thirteen and a half minutes of filming) throughout each film. This interval of forty frames constituted our unit of time for the measurement of velocity of cell displacement. The linear distance between the positions of a given cell on two consecutive stills is one velocity reading. It should be noted that this method of measuring velocity has two sources of bias. It tends to underestimate the true velocity because deviations from a linear course occurring between the two stills are disregarded; the avoidance of erroneous interpretation resulting from this defect will be discussed later. It tends also on the average to overestimate the true

TABLE I

Data on films.

Films 5 and 6 were taken successively on the same culture.

Film	Explants	Hours of filming	Number of cells at beginning		Number of cells after 4 hours		Total cells studied
			In field	Being studied	In field	Being studied	
1	Double	6.2	65	25	160	40	42
2	»	7.1	100	36	200	20	46
3	»	5.8	65	25	200	9	33
4	»	5.3	200	48	400	11	53
5	Single	8.0	19	19	70	22	39
6	»	4.0	65	27	130	20	33

velocity when this is very low because the recorded movement cannot be less than that due to errors of measurement.

About forty cells from each film (see Table I), a total of 246 cells, were followed for varying lengths of time, each cell providing an average of 13 to 14 velocity readings. These observed cells were not taken at random. Cells in mitosis or immediately after were excluded. In order to be chosen a cell had to be distinguishable as an individual continuously over a period. Furthermore, its relations to neighbouring cells had to be fairly clear. These considerations led to certain biases in sampling. Late in the course of filming the double-explant cultures optical difficulties usually arose, owing to an increase in the amount of exudate in the fluid area. Optical difficulties often occurred also close to the explant. Furthermore, when very closely packed in sheets, the individuality of cells tended to be indistinct. Cells in all these situations are under-represented in our sample. An indication of the proportion of cells sampled is given in Table I, which records the estimated total number of cells in the field at the beginning of filming, and again after 4 hours, and the number of cells whose velocity was under observation at these times.

Since the use of the raw figures for velocity led to very skewed statistical distributions, in all the analyses which follow a transformation to log (velocity $+1$) has been used.

RESULTS

After a few hours of incubation cells start to emerge from the explant. In the area adjacent to the explant where the clot has been removed so as to leave a fluid-filled space, the cells advance in a single plane, on the interface between medium and cover slip. According to Weiss (13) the interface may actually be between the medium and an exudate from the explant which coats the cover slip, but we were unable to detect any indication of such a

coat by our methods of observation. The cells in the liquid area show a strong tendency to remain confined to this interface; that is to say, one cell rarely moves across the surface of another. This two-dimensional tendency, which we term "monolayering," will be analysed in a subsequent paper. Most cells are in contact at their lateral edges with one or more of their neighbours, so that when outgrowth is well established the pattern of cells in the liquid area appears at any moment as an irregular two-dimensional meshwork. The cells are more closely packed near the explant, and peripherally there are usually a few isolated cells. Filming of the double cultures usually started when only a fringe of cells bordered each explant leaving an empty space between the two explants, and ended soon after the two zones of outgrowth had made contact with each other.

The movement of this two-dimensional array of cells out from each explant is by no means orderly, in the sense that all cells are moving in the same direction with the same velocity. The arrangement of the cells is continually shifting, and this can be roughly indicated as follows. Taking a cell at random in one of the films, its six nearest neighbours are noted. The fraction of these which are still amongst its six nearest neighbours after an interval representing one hour or two hours of further culture constitutes an index of the orderliness of movement. Twenty-seven estimates after one hour gave a mean index of 0.58 ± 0.09; and twenty estimates after two hours a mean index of 0.39 ± 0.08.

That the arrangement of the cells is shifting to this extent is partly due to movement of the cells in a variety of directions, but this variability in direction of movement will not be analysed here. It is also partly due to the variability of the velocities with which cells move. The purpose of the present paper is to begin the analysis, in terms of cell interaction, of this variety of cell velocities. It is perhaps worth emphasising that the variability of velocity is very great. Coefficients of variation of samples of velocities are usually around 50 per cent (standard deviation as a percentage of mean) for readings transformed to logs, and higher still, though less meaningful because of the skewed distribution, for raw readings. This variability is to be found between different cells measured simultaneously in one culture; for instance, a range of velocity of 0 to 250 μ per hour was commonly found at any one time; and a coefficient of variation based on the variance of readings taken simultaneously in one culture from 17 cells, and repeated at 20 consecutive intervals of time, was 56.7 per cent (transformed velocity; 320 degrees of freedom). The variability is to be found also in velocity fluctuations of an individual cell during the course of culture; the time-course of velocity of a

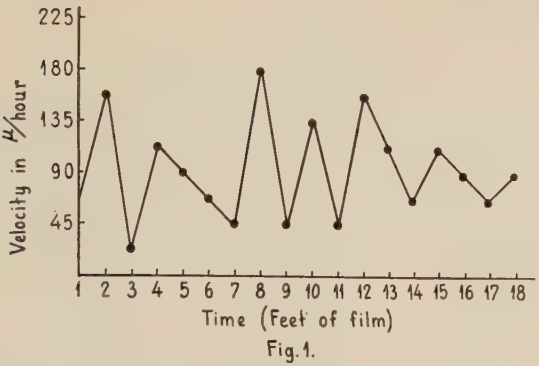

Fig. 1. Example of the variability in velocity of a single cell during the course of culture.

representative cell illustrates this (Fig. 1); and a coefficient of variation based on the variance within the record of each of 60 cells observed during 20 consecutive readings was 50.2 per cent (transformed velocity; 1 140 degrees of freedom).

As a first step in the analysis of this high variability of velocity, we consider the velocity of any cell in relation to the number of other cells in contact with it. We obtained this number of contiguous cells both at the start and at the finish of the standard length of film (40 frames) from which, in the way already described, each velocity reading was made. The mean of these two determinations of number of touching cells constitutes the *contact number* for the given velocity reading. Changes in mutual contacts are sufficiently slow for the great majority of them to appear as changes in recorded contact number.

Since the distribution of the raw velocity readings at any given contact number was highly skewed, a transformation of velocity to log (velocity + 1) was used for the statistical treatment. This transformation makes the distributions of velocities substantially normal, as shown by plotting them as probits. In Table II mean transformed velocities at different contact numbers are recorded and also these means converted into terms of μ per hour. The latter are plotted against contact number in Fig. 2. There appears from these figures to be an inverse relationship between contact number and velocity. At the highest contact numbers the relationship is not evident, but sample size is here inadequate and there is also some difficulty in determining precise contact number.

In order to test the significance of this apparent inverse relationship a

TABLE II

Contact number and velocity.

Contact number	No. of readings	Transformed Velocity, i.e., log (velocity +1).		
		Mean	Standard deviation	Mean, converted to μ/hour
0	409	0.453	0.217	82.7
0.5	185	0.492	0.231	94.7
1	378	0.436	0.215	77.8
1.5	315	0.440	0.224	78.9
2	476	0.411	0.210	70.9
2.5	360	0.412	0.204	71.2
3	344	0.378	0.199	62.5
3.5	273	0.366	0.191	59.5
4	254	0.327	0.184	50.5
4.5	156	0.341	0.182	53.7
5	119	0.282	0.191	41.1
5.5	43	0.275	0.174	39.8
6	18	0.295	0.217	43.7
6.5	12	0.318	0.162	48.6
7	3	0.360	0.102	57.0

straight regression line has been fitted to the data. It will be seen from Table II that as between the different contact numbers there is some dependence of standard deviation on mean; but the standard deviations do not differ seriously enough to invalidate the calculation. The straight line calculated for the whole of the transformed data is a fairly good fit; the variance around the regression line between the means of the groups specified by the different contact numbers is not significantly higher than the variance within the groups ($P = 0.1$; appendix 1). The regression coefficient calculated for the whole of the transformed data is -0.0326 ± 0.0024. This coefficient is highly significantly different from zero ($t = 13.6$ for 3 343 degrees of freedom; $P < 0.001$; appendix 2). The inverse relationship found between velocity and contact number is therefore statistically valid.

We have so far treated the data en masse. The regression coefficient for each culture (grouping together therefore films 5 and 6) is given separately in Table III, with its standard error, and t for its difference from zero. The expected velocity (transformed) when contact number is 0, and the product moment correlation coefficient (r), are also tabulated. In the last line the

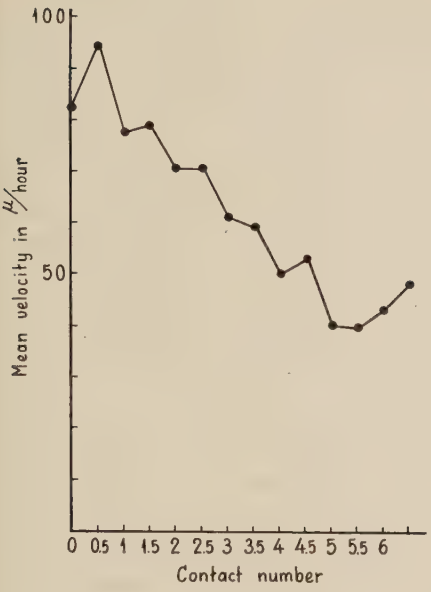

Fig. 2.

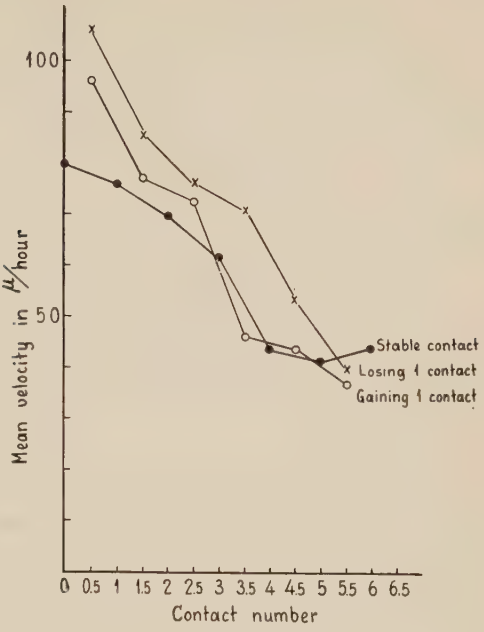

Fig. 3.

Fig. 2. Mean velocities, all readings, plotted according to contact number.
Fig. 3. Mean velocities according to contact number, • of readings where the contact number remained stable during the observation of velocity, ○ where a contact was gained during observation, and × where a contact was lost.

same parameters are given for all the data, after the variance between the different cultures has been eliminated. Films 1, 2 and 3 show a regression of velocity on contact number which is highly significantly different from zero ($P < 0.01$ in all). In the light of this the regression in films 5 and 6 ($P = 0.05$) is probably to be taken as significant. It will be shown later that when an obscuring factor is removed from the data of film 4 this film too probably has a significant regression; though it must be noted that this culture is somewhat unlike the rest in that it tends towards a "mesothelial" style of growth. The correlation coefficients of Table III show that the relationship between velocity and contact number, though significant, is not a strong one.

In the last column of Table III are mean velocities adjusted to the values expected at the common mean contact number of all the data, using the average within-film regression coefficient of the last line of Table III. These adjusted means diverge from each other more than would be expected by chance judging from the variability within the individual films. When the

TABLE III

Regression of velocity on contact number in the different films.

Film	Regression coefficient and standard error	Degrees of freedom	t for difference from zero	Expected velocity 0 contact	r	Adjusted mean velocity
1	-0.042 ± 0.004	903	9.89	0.484	−0.31	0.379
2	-0.028 ± 0.006	751	4.87	0.449	−0.18	0.387
3	-0.035 ± 0.009	330	3.74	0.521	−0.20	0.444
4	-0.007 ± 0.006	511	1.24	0.372	−0.05	0.370
5 and 6	-0.012 ± 0.006	840	2.02	0.481	−0.07	0.444
Within cultures	-0.027 ± 0.003	3 339	10.11	0.462	−0.17	

mean square appropriate for testing the adjusted means is compared with the mean square within films by analysis of covariance, they differ significantly ($P < 0.001$; appendix 3). Similarly, the regression coefficients differ from each other more than is to be expected by chance; the mean square due to differences between the regression coefficients is significantly bigger than the appropriate error mean square ($P < 0.001$; appendix 4). There is individuality therefore in both the mean speed and the slope of the regression line between different cultures. Films 5 and 6, taken consecutively on the same culture, when compared with each other, differ significantly in their adjusted mean velocities, film 6 being the faster, but not in the slopes of their regression lines.

For a full analysis of variations between cultures much more extensive data are required, and we will not pursue the problems here. It is established, however, that in these cultures an inverse relationship, though a varying one, holds between contact number and velocity, both measured in the way described. The further interpretation of this inverse relationship is, however, by no means straightforward. It requires the examination and negotiation of several possible pitfalls; and in doing this we have for simplicity treated the data as a whole, without considering individual films.

The time interval in velocity estimation. The first point we have to examine is that the inverse relationship between contact number and velocity may be quite trivial in that it may have arisen entirely out of our method of measurement. In estimating velocity our observations of the position of any cell were spaced at intervals of time. Any irregularity in the course taken by a cell during the interval between observations is thus disregarded. Consequently,

if irregularity of the course taken, i.e., rate of change of direction, increases, the net movement recorded between two observations will correspondingly underestimate the true distance moved. It is clear therefore that if contact number is correlated with rate of change of direction it might thereby be inversely correlated with observed velocity, even though true velocity remained constant.

A check on this possible fallacy of interpretation can be obtained by increasing the frequency of observation and seeing whether this affects the inverse relationship between velocity and contact number. In the first place we estimated the velocity of a sample of cells, measuring the linear distance between their positions recorded every 20 frames (6.7 minutes of culture time) instead of every 40 frames. 362 readings from 33 cells of all 6 films were made, measured directly on the ground-glass screen of a film-viewer instead of on stills. There proved to be no evidence whatever that the decreased interval of observation tended to abolish the relationship of velocity and contact number. A regression line fitted to the new data (transformed) had on the contrary a slightly steeper slope ($b = -0.040 \pm 0.011$) than that already recorded from the measurements at 40 frame intervals ($b = -0.033 \pm 0.002$), though the difference is not significant. As would be expected, since more irregularities of movement are included, mean velocities tended to be a little higher in the 20 frame sample than in the 40 frame sample.

A second check was made on a sample of 25 cells, measuring velocity by a different method. A known length of film was projected slowly on to paper, and the course followed by a particular cell was traced in pencil. The total distance travelled was then measured with a map measurer, and converted to absolute units. Each cell was followed for an average of 20 feet of film ($4\ ^1/_2$ hours of culture time). This method does not achieve the maximum continuity of observation theoretically obtainable from a film, but it provides the equivalent of a much increased frequency of observation, we estimate at least once every 5 frames, as compared with the standard method of a still every 40 frames. Data bearing on the error of estimation by such a tracing method are found in Hick (3) and Vince (12). The velocities obtained were the means for given cells during a relatively long period of observation, and as such could not for statistical testing be transformed to logs, and compared to the transformed individual readings obtained by the usual method. Mean velocities could, however, be calculated for the same cells during the same period, from measurements made in the usual way, and the two estimates of velocity for each cell compared. They proved to be very similar ($r = 0.95$ for the 25 cells), though as expected those estimated by the contin-

uous projection method averaged rather higher. Mean contact numbers of these cells for the period during which velocity was estimated varied from 0 to 4.5. Ten cells with mean contact number between 0 and 1.5 (average 0.38) had a mean velocity of 105.7 μ/hour measured by projection, 91.4 μ/hour measured by stills at 40 frame intervals; and 15 cells with mean contact number greater than 1.5 (average 2.52) had a mean velocity of 86.0 μ per hour and 78.8 μ per hour respectively. There is therefore no sign that the projection method diminished the inverse relationship between velocity and contact number; indeed, as perhaps with the 20-frame analysis, it somewhat intensified the relationship, the regression of readings by the still method on readings by the projection method having a coefficient of 0.73 ± 0.05.

These results incidentally show that three quite distinct methods of measuring velocity produce concordant results. Our main interest in them is, however, that they indicate that observed velocity is not simply an outcome of rate of change of direction in these cultures. Little would be gained by attempting more thorough confirmation by further increasing the frequency of observation. However short the time intervals, there may always be irregularities of path which have a frequency considerably greater than the frequency of observation. Consequently in defining cell velocity, when it is to be measured by a discontinuous method as here, some arbitrary limit of observation frequency must be fixed. Such a limit has some rationality because the smaller the irregularities of observed path the more they represent random fluctuations of the nucleus or nucleolus within the cell, and the less they represent the correlated movement of the majority of cell components which constitutes cell displacement. Such random fluctuations are becoming obtrusive in our method of tracing a cell during projection.

Effect of age of culture. A relationship might be expected between cell velocity and the length of time which has elapsed since the culture was prepared, in view of the known decline in "growth rate" of cultures with age. Such a relationship might also be the result of intrinsically faster-moving cells leaving the explant early, to be followed later by slower ones. It could be important to our hypothesis because at the beginning of the period of filming cells are relatively sparse and contact numbers consequently low, while by the end of the period there are many cells with high contact numbers. Hence our sample of observations with low contact numbers might be, and indeed obviously is, biassed towards the early stages of culture, our sample of high contact numbers towards late. But since "early" cells may be moving relatively fast, and "late" cells relatively slowly, for reasons un-

connected with contact number, the observed decline of velocity with increasing contact number might really be a decline with age of culture.

To test this possibility we have compared, in each film, velocity readings obtained during the first 80 minutes of filming with those obtained in the last 80 minutes. (Films 5 and 6, made on the same culture, have been treated separately in view of the significantly higher mean speed of the area of this culture used for film 6 as compared with the area used for film 5.) The two groups of observations are separated by an average interval, for all the films, of $2\ ^1/_2$ hours of culture. The earlier group has a mean velocity (transformed) of 0.415, and a mean contact number of 1.93; the later 0.387 and 2.41 respectively. The difference seems quite inadequate to account for the overall contact-velocity relationship already found. It was tested by analysis of covariance. The two group mean velocities, adjusted to their common mean contact number, became 0.410 for the earlier, 0.394 for the later. This difference is not significant ($P = 0.1$–0.2; see appendix 5). Furthermore, when the within-group regression coefficient for velocity on contact number is calculated for the earlier and later groups combined, the differences between the two group means being eliminated, it proves to be highly significant ($b = -0.024 \pm 0.004$, $t = 5.9$ for 1 352 degrees of freedom, $P < 0.001$). It is clear that the contact-velocity relationship exists quite independently of any ageing of the culture which may occur during the relatively brief period of our filming.

Number of neighbouring cells. A more serious potential fallacy in interpreting the contact-velocity relationship is that the number of contacts may be simply a reflection of the number of neighbouring cells. In terms of our original hypothesis, if we assume for the moment that velocity is the dependent variable, it may not be contacts with other cells which reduce velocity, but some inhibitory substance released by other cells, the concentration of the substance varying with the density of neighbouring cell population. The relationship of the three variables, velocity, contact number and number of neighbouring cells has therefore been investigated by a multiple regression analysis (see appendix 6). Number of neighbouring cells is defined as the number of cells whose nuclei fall within a circle of radius 100 μ drawn round the nucleus of the observed cell. The range of readings of number of neighbouring cells was 0 to 17, mean 6.8. Since number of neighbours could not be recorded for cells near the edge of the field, our sample of readings was reduced to 2 309. Contact number was, as expected, highly correlated with number of neighbours ($r = 0.81$); and both were negatively correlated with velocity (contact number, $r = -0.29$; number of neighbours, $r =$

-0.23). The partial regression coefficient of velocity on contact number, number of neighbours being held constant, was -0.045 ± 0.005, which is highly significant ($t = 9.00$ for 2 306 degrees of freedom, $P < 0.001$). The partial regression coefficient of velocity on number of neighbours, contact number being held constant, was $+0.0007 \pm 0.0013$, which is obviously not significantly different from zero ($t = 0.523$ for 2 306 degrees of freedom, $P = 0.6$). The partial correlation coefficients are -0.18 for velocity and contact, and $+0.01$ for velocity and number of neighbours. We may conclude therefore that, in our data as a whole, the number of neighbouring cells is quite without any independent relationship with velocity; its relationship to velocity is entirely accounted for by its relationship to contact number. The possible fallacy stated at the beginning of this section proves therefore to have no substance.

Distance from explant. On the whole cells with the lower contact numbers and higher speeds tend to lie peripherally in the zone of outwandering cells, those with higher contact numbers and lower speeds being found nearer the explant. It is consequently possible that confounding of contact number with distance from explant may be misleading us. We have therefore divided the frame of each film into a series of zones 200 μ wide, successively further from each explant. In the double cultures there are two such zones for each explant, and a third, central, zone equidistant from both explants, except in film 4 where there is insufficient space for the central zone. Velocity readings were taken only up to the point when the opposing groups of cells started to meet. In the single culture there are three zones of similar width.

Taking the data as a whole, and standardising for contact number by analysis of covariance in the usual way, we find that the zone furthest from the explant gives appreciably higher readings than do the inner two zones. The adjusted mean velocities are, proceeding outwards, 0.402, 0.394 and 0.444 (see appendix 7). The higher speed in the outermost zone is actually due to one film, film 2, in which the superiority of this zone is very strongly marked. The other films give no evidence that velocity tends to be higher in the outermost zone. They suggest rather that there are microclimates, not related to distance from the explant, where speeds are unusually fast or slow; but this possibility must be investigated on more extensive data. The actual distribution of speeds by zones in the present data, however, is such as to lead to confounding with the contact-velocity relation. It is necessary therefore to calculate the within-zone regression coefficient, after eliminating the variance due to differences between the zone means. The coefficient is 0.032 ± 0.003, substantially the same as that for the whole data as previously

calculated. This within-zone coefficient is highly significantly different from zero ($t = 11.1$ with 2 508 degrees of freedom; $P < 0.001$). Furthermore, the significance of the regression coefficients of the individual cultures, including that of film 2, is unimpaired by making the analysis on the within-zone sums of squares and products for each film separately. In the case of film 4, where the adjusted mean speed is slower in the peripheral than in the central zone, the within-zone regression coefficient is significantly different from zero ($b = -0.019 \pm 0.09$, $t = 2.14$ with 264 degrees of freedom, $P = 0.02$–0.05) while, as already recorded, all the data from this film being used and the central-peripheral disposition being disregarded the regression was not significant. We conclude therefore that our hypothesis of an immediate relationship between contact number and velocity is not vitiated by confounding with the effects of distance from explant.

Experimental increase of contact number. Examination of the more obvious sources of confounding with other variables, in the previous sections of this paper, have disclosed no evidence against the hypothesis of an immediate relationship between velocity and contact number. The hypothesis would be tested more severely if we could vary experimentally either contact number or velocity. Contact number can be increased very simply by increasing the density of population of the cells. In our double cultures the relatively fast-moving peripheral cells with low contact number undergo an increase in contact number when the outgrowths of the two explants fuse.

To carry out such a test we have examined 27 cells, from films 2, 3 and 4 (the opposing outgrowths in film 1 do not fuse sufficiently for our purposes). These cells were the most peripheral of the outgrowing sheets of cells, and therefore the ones which undergo the greatest average increase of contact number when the outgrowths meet. A point of time was selected when it was considered that fusion of the opposing outgrowths had begun and nine consecutive velocity readings were obtained for each cell before and after that point (except in film 4 where only 4 readings were available before the fusion point). The mean velocities of the two groups, before fusion and after fusion, were 0.501 and 0.449 respectively, and the mean contact numbers 1.56 and 2.27 respectively. When the variance of the data is analysed, the mean square between groups significantly exceeded the mean square within groups; for velocities, $t = 2.67$ for 444 degrees of freedom $P = 0.01$, and for contact numbers $t = 6.99$ for 444 degrees of freedom, $P < 0.001$. The cells therefore differ significantly before and after fusion in velocity and contact number. When the mean velocities are adjusted by the within-group regression coefficient to values expected at the common mean contact

number, they become 0.483 before fusion and 0.464 after. While the difference in velocity before and after fusion is not entirely abolished by allowing for the difference in contact number, it can now be entirely accounted for by sampling error. Tested by analysis of covariance t for the difference between adjusted means is 0.95 with 443 degrees of freedom, $P = 0.3$ to 0.4, which is quite insignificant. The regression coefficients for velocity on contact number do not differ significantly before and after fusion ($t = 0.85$ with 442 degrees of freedom, $P = 0.4$).

Further information about the effects of fusion can be obtained in another way. Sums of squares and products for velocity, contact number and time were worked out separately for each cell, and summed in the two groups (before and after fusion) so as to eliminate the variance between cells. Before fusion b for contact on time was -0.036 ± 0.018 (for difference from zero $t = 2.01$ with 175 degrees of freedom, $P = 0.05$), while b for velocity on time was -0.003 ± 0.006 ($t = 0.51$, $P = 0.7$). Before fusion there is therefore a slight but perhaps significant decrease in contact number (attributable to the breaking free of cells from the initially rather compact outgrowth), but no significant change in velocity can be detected. (It will be recalled that a relatively large change of contact number is associated with a small change of velocity.) After fusion b for contact on time was $+0.155 \pm 0.014$ ($t = 11.0$ with 215 degrees of freedom, $P < 0.001$) and b for velocity on time was -0.009 ± 0.005 ($t = 1.97$ with 215 degrees of freedom, $P = 0.05$). After the fusion point therefore there is a considerable and highly significant increase of contact number with time, accompanied by a decrease in speed which in view of the previous analysis we may regard as significant.

We conclude therefore that velocity diminishes with the increased contact number induced by fusion of outgrowths. While the experiment is not rigidly controlled, it is difficult to suggest, particularly in view of our previous analysis, what significant influence other than increased contact number could have been introduced. Confidence in the existence of an immediate relationship between contact number and velocity is therefore strengthened, and it is made probable that velocity is the dependent variable. We have not, however, excluded the possibility that a reciprocal effect of velocity on contact number may at times play some part, slower cells having perhaps more chance than faster cells of forming and maintaining multiple contacts.

Contact change. The curve of contact number against velocity shown in Fig. 2 visibly departs from linearity in one respect. The mean velocities of cells whose contact number is expressed as a fraction tend to be unexpectedly high as compared with the mean velocities when the contact

Table IV

Effects of gaining or losing a contact.

Contact change	Regression coefficient	and	Standard error	Degrees of freedom	Difference from zero t	Expected velocity 0 contact
0	−0.046	±	0.004	1 747	12.7	0.480
+1	−0.057	±	0.005	608	10.8	0.544
−1	−0.089	±	0.007	566	13.2	0.658

number is a whole number. The curve connecting the means therefore tends to have a zig-zag shape. A fractional contact number invariably represents, for each cell thus classified, a loss or a gain of an odd number of contacts (usually of one contact) during the period through which the distance travelled was recorded (contact number being defined as the mean of the numbers of contacts before and after the period of measurement). The whole contact numbers mostly represent no change in numbers of contacts through the period concerned, though a few of them represent the loss or gain of an even number of contacts. The implication is therefore that there is a transitory increment of velocity associated with immediate loss or gain of a contact.

In order to test whether we have here a second significant relationship between contact and velocity, we have separated the readings where one contact is gained during the period of velocity measurement and those where one contact is lost, and compared them with those where contact number remains stable. Data on these three groups are given in Table IV. It will be noted that the significant regression when contact number is stable excludes the possibility that the relationship between contact number and velocity is really due to confounding with a relationship between contact change and velocity. In Fig. 3 the mean velocities (expressed as μ/hour) for the different contact numbers are plotted for each of the three groups of data.

In comparing these groups the mean group velocities were as usual adjusted to correspond to the common mean contact number, and the adjusted means tested by analysis of covariance. Taking first the stable and the gaining-one groups, the adjusted means were 0.386 and 0.422 respectively, the difference between them being highly significant ($P < 0.001$; appendix 8).

The slopes of the two regression lines did not, however, differ significantly ($P = 0.1$–0.5; appendix 8). Comparing the stable contact and the losing-one contact groups, the adjusted means were 0.384 and 0.459 respectively, again highly significantly different ($P < 0.001$; appendix 9). The slope of the regression line of the losing-one contact group was significantly steeper than that of the stable contact group (< 0.001; appendix 9). Finally, comparing the gaining-one group with the losing-one group, adjusted means were 0.401 and 0.437 respectively, which are significantly different ($P < 0.01$, appendix 10). The slope of the losing-one regression line was significantly steeper than that of the gaining-one regression line ($P < 0.001$; appendix 10).

We may conclude therefore that there are short-term changes in velocity associated with the act of gaining or losing a contact which are distinct from the relationship already established between contact number and velocity. Taking an average cell with a certain contact number, the immediate correlate of gaining a contact will be an acceleration, though the long-term correlate of the additional contact will be a diminution of speed. The act of losing a contact is associated with a transitory acceleration considerably greater than that to be expected from the relationship between contact number and velocity, and greater also than that associated with gaining a contact. The transitory increment of velocity when a contact is lost is greater the smaller the contact number.

Two hypotheses are possible about the causal relations involved. Increase in velocity might be supposed to raise the chances of a cell making a contact, or in other circumstances to increase the chances of a cell parting from one with which it is in contact. On the other hand, when a contact is made the cells concerned may tend to run together, and when a contact is broken the cells may tend to spring apart. Observation lends strong support to the second hypothesis, demonstrating that acceleration usually succeeds the contact change. Cells may sometimes be seen to move towards each other with a transitory acceleration immediately after they have touched. Much more frequently they may be seen to draw slowly away from each other, often pulling out a strand of cytoplasm between them; then the moment their mutual connection parts they jerk away. It would require a more refined analysis than we have yet attempted to put this temporal relationship between the acceleration and the moment of making or breaking a contact into quantitative form. The qualitative observation is, however, clear enough to establish that the change of contact causes change of speed; though again it is possible that to some extent the converse relationship, change of speed causing change of contact, also plays a part.

DISCUSSION

We have failed to discover evidence against our two hypotheses: (a) that the velocity of movement of a fibroblast is depressed by an established contact with another fibroblast, and (b) that it is momentarily increased by the making or breaking of a contact. We can, therefore, provisionally consider these hypotheses as confirmed. How does contact produce these effects? We suggest that an important part of the explanation is likely to be in terms of adhesion between cells, a phenomenon whose significance in the architecture of tissue cultures has been particularly emphasised by Levi (7) and Lewis (8).

The contacts formed between fibroblasts in tissue cultures are well-known to be extremely intimate, though they often involve only a small part of the surfaces of the cells concerned. Direct observation of living or fixed preparations rarely enables one to say where one cell ends and another begins, though it at once becomes clear when one of the cells is killed by severe damage to its nucleus (2, 6). These connections are probably always temporary in an actively growing culture, but they can be very firm while they last; they are in fact adhesions, as Kredel's microdissection experiments demonstrate. Similar transitory adhesions occur between cell and cover slip. Probably not everything we call a "contact" is in fact an adhesion; and no doubt adhesions are of varying strength, so that we are greatly oversimplifying when we treat all contacts as equal. Our methods only detect the average effect of adhesion.

Adhesion between cells may be supposed to produce its effect on cell velocity as follows. The direction of movement of a cell is normally not completely correlated with that of its neighbours. When two cells are in contact their direction of movement is liable to be incompatible. As a result of the adhesion between them, they push or pull against each other to some extent; some of the energy which would normally go into movement is thereby dissipated or becomes potential energy of elastic tension between the cells, and their speed of movement tends to be diminished. With increasing number of contacts, the amount of restraint imposed on a cell increases. When an adhesion breaks, the release of potential energy stored as elastic tension produces the observed sudden acceleration. The establishment of an adhesion, it may be assumed, involves a rapid but limited spread of the area of interface between the two cells, which tends to draw them together, accounting for the momentary acceleration on making contact.

While this effect of adhesion is, we consider, the most important way in which contact affects velocity in these cultures, we may have to reckon also with a phenomenon already mentioned, that of "monolayering." As will be shown in a subsequent paper, fibroblasts tend not to move over each other's surfaces. This presumably means that when a contact is formed, further movement in the direction of the contacting cell tends to be inhibited. The initial spread of the area of mutual contact which we have postulated to explain the apparent drawing together of cells must therefore be of a strictly limited extent. Such monolayering must, it would seem, be important in influencing the direction taken by a fibroblast; but it is not so certain that it influences the velocity of a fibroblast; though clearly this is a possibility. A further possibility which must be borne in mind, since Holtfreter (5) has observed it in amphibian cells, is that contact with other cells tends to depress the entire locomotory mechanisms of a cell.

We will not at present consider the precise role of the contact-speed effects which we have demonstrated in the behaviour of fibroblast cultures or of fibroblasts within the organism. We ought perhaps to emphasise, however, that although other correlations with velocity have proved to be slight or negligible in the present work, they may in some circumstances prove to be important. The analyses used in the present work were intended merely to eliminate the effects of confounding of other influences with those of contact. They are not themselves a fair test of the importance of these other influences for fibroblast behaviour.

SUMMARY

1. Heart fibroblasts from 7–10 day chicks were cultured in liquid medium and observed by time-lapse cinemicrography.

2. Analysis of the films showed a significant inverse relationship between the speed of movement of a cell and the number of other cells with which it was in contact during the observed movement.

3. The possibility was excluded that this correlation was spurious, in that it might be due to confounding with age in culture, with density of surrounding cell population or with distance of the observed cell from the explant.

4. The numbers of contacts between cells was experimentally influenced by placing two explants close together and allowing their outgrowths to fuse. Observation of the peripheral cells of these outgrowths before and after fusion showed that an increased number of contacts caused a decrease in speed.

5. A momentary increase of speed was also correlated with, and probably caused by, the act both of gaining and of losing a contact with another cell.

6. It is suggested that the slowing of fibroblasts by contact with others is mainly to be ascribed to the effects of adhesions between the cells; and the acceleration by change of contact, to the effects of development or rupture of such adhesions.

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APPENDICES

1. Test of linearity. ((10), p. 382.) Sums of squares of transformed velocities: total 150.769; within contact-number groups 142.006 (3 330 degrees of freedom); between groups 8.763; due to regression 7.914; between groups, deviations from regression 0.849 (13 degrees of freedom). Mean squares: within groups 0.0426; between groups, deviations from regression 0.0653. $F = 1.53$, $P = 0.1$.

2. Regression of velocity on contact number, all data. ((10), p. 117.) $X =$ contact number. $Y =$ transformed velocity. $N = 3\,345$. $\Sigma(X - \bar{X})^2 = 7\,462.8$. $\Sigma(Y - \bar{Y})^2 = 150.769$. $\Sigma(X - \bar{X})(Y - \bar{Y}) = -243.024$. $b = -0.0326$. Sum of squares of Y due to regression 7.914; residual sum of squares around regression line 142.855; mean square 0.0427. Standard deviation of $b = 0.00239$. $b/Sb = 13.61$ with 3 343 degrees of freedom, $P < 0.001$.

3. Difference between means of cultures. ((10), p. 318.) Sums of squares of transformed velocities: within cultures 144.097; due to regression within cultures 4.279; residual within cultures 139.818 (3 339 degrees of freedom); total residual 142.855; difference for testing adjusted means, 3.036 (4 degrees of freedom). Mean squares: residual within cultures 0.0419; for testing adjusted means 0.759. $F = 18.1$, $P < 0.001$.

4. Difference between regression coefficients of cultures. ((10), p. 327.) Sums of squares of transformed velocities: residual, within cultures 139.818; residual from individual culture regressions 138.632 (3 335 degrees of freedom); due to differences of culture regressions 1.187 (4 degrees of freedom). Mean squares: from individual regressions 0.0416; due to differences of regressions 0.297. $F = 7.14$, $P < 0.001$.

5. Age of culture. ((10), p. 330.) Comparison of group ($N = 843$) of early with group ($N = 512$) of late velocity readings. Sums of squares of transformed velocities: total 58.074; due to total regression 1.616; total residual 56.458; within groups 57.834; due to regression within groups 1.457; residual within groups 56.377 (1 352 degrees of freedom); difference for testing adjusted means 0.0810 (1 degree of freedom). Mean squares: residual within groups 0.0417; for testing adjusted means 0.0810. $t = 1.39$, $P > 0.1$.

6. Number of neighbours. Multiple regression analysis ((10), p. 340) of number of neighbours (W), contact number (X) and transformed velocity (Y). $N = 2\,309$. $\Sigma(W - \bar{W})^2 = 73\,640$; $\Sigma(X - \bar{X})^2 = 5\,261.3$; $\Sigma(Y - \bar{Y})^2 = 112.09$; $\Sigma(Y - \bar{Y})(W - \bar{W}) = -671.86$; $\Sigma(X - \bar{X})(W - \bar{W}) = 16\,039$; $\Sigma(X - \bar{X})(Y - \bar{Y}) = -225.82$. $r_{wx} = 0.815$; $r_{wy} = -0.234$; $r_{xy} = -0.294$. Standard $b_{yx \cdot w} = -0.308$; standard $b_{yw \cdot x} = 0.0173$. $R^2 = 0.0866$. Standard error of standard $b = 0.0343$. True $b_{yx \cdot w} = -0.0450 \pm 0.0050$; true $b_{yw \cdot x} = +0.00070 \pm 0.00134$.

7. Distance from explant. ((10), p. 318.) Three groups of readings from three successively more peripheral zones ($N = 697$, 1 380 and 436 respectively). Sums of squares of transformed velocities: total 116.442; due to total regression 7.494; total residual 108.947; within groups 113.476; due to regression within groups 5.292; residual within groups 108.184 (2 508 degrees of freedom); difference for testing adjusted means 0.7629 (2 degrees of freedom). Mean squares: residual within groups 0.0431; for testing adjusted means 0.3815. $F = 8.85$, $P < 0.001$.

8. Effect of gaining a contact. ((10), p. 318.) Group of readings with constant contact ($N - 1\,749$) compared with group where one contact is gained ($N = 610$). Sums of squares of transformed velocities; total 100.978; due to total regression 9.723; total residual 91.254; within groups 100.926; due to regression within groups 10.264; residual within groups 90.662 (2 356 degrees of freedom); difference for testing adjusted means 0.593 (1 degree of freedom). Mean squares: residual within groups 0.0385; for testing adjusted means 0.593. $t = 3.92$, $P < 0.001$.

Sums of squares of transformed velocities: residual from individual group regressions 90.546 (2 355 degrees of freedom); difference from residual within groups due to differences of group regressions 0.116 (1 degree of freedom). Mean squares: from individual regressions 0.0385; due to differences of regression 0.116. $t = 1.73$, $P = 0.05 - 0.1$.

9. Effect of losing a contact. ((10), p. 318.) Group of readings with constant contact ($N = 1749$) compared with group where one contact is lost ($N = 568$). Sums of squares of transformed velocities: total 106.840; due to total regression 10.539; total residual 96.301; within groups 106.052; due to regression within groups 12.091; residual within groups 93.961 (2 314 degrees of freedom); difference for testing adjusted means 2.340 (1 degree of freedom). Mean squares: residual within groups 0.0406; for testing adjusted means 2.340. $t = 7.59$, $P < 0.001$.

Sums of squares of transformed velocities: residual, from individual group regressions, 92.6869 (2 313 degrees of freedom); difference from residual within groups, due to differences of group regressions, 1.274 (1 degree of freedom). Mean squares: from individual regressions 0.0401; due to differences of regressions 1.274. $t = 5.64$, $P < 0.001$.

10. Gaining a contact compared with losing one. ((10), p. 318.) Previous "gaining-one" and "losing-one" groups compared. Sums of squares of transformed velocities: total 54.074; due to total regression 10.256; total residual 43.819; within groups 53.769; due to regression within groups 10.331; residual within groups 42.438 (1 175 degrees of freedom); difference for testing adjusted means 0.380 (1 degree of freedom). Mean squares: residual within groups 0.0370; for testing adjusted means 0.380; $t = 3.21$, $P = 0.01 - 0.001$.

Sums of squares of transformed velocities: residual, from individual group regressions 42.932 (1 174 degrees of freedom); difference from residual within groups,

due to differences of group regressions, 0.506 (1 degree of freedom). Mean squares: from individual regressions 0.0366; due to differences of regressions 0.506; $t = 3.72$, $P < 0.001$.

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QUANTITATIVE STUDIES ON THE DEPENDENCE OF CELL MORPHOLOGY AND MOTILITY UPON THE FINE STRUCTURE OF THE MEDIUM IN TISSUE CULTURE[1]

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Cells have traditionally been identified pictorially. This has become common practice because quantitative information adequate for proper classification has been lacking. Such a visual approach obscures the fact that a single cell can and often does pass from one morphological state to another. There are two distinct types of transformation: 1) a developmental differentiation which is irreversible, and 2) a transitory, reversible adaptation to environmental conditions, termed "modulation" by Weiss (21, 24). Modulations from one cell form to another have previously been noted in tissue culture for almost every cell type. For example, the following cells have been observed to transform into macrophages: fibrocytes (2, 7, 10, 14), Schwann cells (22, 26), muscle cells (6), and epithelial cells (3, 17). Reverse transformations have also been observed: macrophages into fibrocytes (4, 9) and epithelial cells into fibrocytes (5, 18).

Weiss (19) found that the morphological character of chick mesenchyme cells growing in tissue culture varied with the plasma content of the medium. In plasma medium of high fibrin content, the cells were long and spindle-shaped, especially along the lines of tension where the fibrin had aggregated to form thick bundles. When the consistency of the medium was changed by increasing fluidity, these cells appeared in a plumper, amoeboid form. In addition, it was noted that cells migrated faster in clots of high fibrin content and along the lines of tension as compared to migration in clots of high liquid content and unoriented matrices, where thick bundles of fibrin were absent. These observations were further elucidated by Weiss (20, 23) in describing nerve outgrowth *in vitro*.

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Proceeding from this information, the present investigation studies on a quantitative level the dependence of cell morphology and motility upon the physical structure of the medium. The tissue culture technique was chosen because it provided the opportunity to introduce isolated and homogeneous cell populations of known history into an environment whose physical constitution can be systematically altered, independently of the cells.

A graded series of plasma clots can be produced by varying the plasma concentration. With increasing plasma concentration, the lateral association of fibrin bundles increases, producing gels of increasing coarseness. The aggregation of fibrin into larger and larger bundles during the coagulation of fibrin clots has been followed with the dark field microscope (1) and in greater detail by means of the electron microscope (16). A second way of obtaining a series of plasma clots of graded texture is the variation of the pH of the clot at the time of coagulation. Hawn and Porter (12) have shown in electron micrographs that the more acid the pH, the greater the degree of lateral aggregation of fibrin, and, thus, the coarser the clot texture.

The present paper is limited to a presentation of the experimental methods and the detailed data of this investigation. The interpretation and a discussion of the biological significance of this work is to be found in a previous publication (25).

MATERIALS AND METHODS

Experimental procedure. Uniform cell strains of chick heart fibrocytes were established from heart explants of eight-day embryos, using the hanging drop tissue culture technique, according to common procedures detailed in Parker (15). The standard clot for the stock cultures consisted of one drop of chicken plasma and one drop of embryonic extract. The plasma used was obtained from fresh chicken blood collected under sterile conditions from the carotid artery without use of anticoagulant. Embryonic extract was prepared from ground-up eight-day embryos diluted with Tyrode's solution to three times the volume of the embryo. Every second or third day the cultures were divided, washed in Tyrode's solution, and transferred into fresh medium. After the fifth passage, the cell strain was considered sufficiently homogeneous for the purposes of the experiment, and subsequent cultures were used as experimental material.

Two series of experiments were made, using standard cell material of uniform fibrocyte cultures while systematically varying the physical constitution of the medium, either by varying the concentration of the plasma in the clot or by varying the pH of the clot.

In the first series of experiments, clots of 10, 30, 50, 70 and 90 per cent plasma were prepared at physiological pH. A plasma-embryonic extract mixture of the proper proportions was first mixed, and two drops of the mixture were quickly pipetted and spread on each of a series of glass cover slips before clotting took place.

After each clot had become firm, an explant from the standard strain was placed on the surface of the clot, and the culture was sealed and incubated at 37.5° C. Thus, all individual clots of the same constitution were prepared from the same mixture. It is important to note that the clot texture was allowed to become established before introducing the tissue explant. Although the formation of fibrin is a progressive reaction and may continue for several hours after a firm clot has first formed, the basic fine structure of the clot is determined by the time the gel has set (13).

The second series of experiments involved a change in the pH of the clot. Clots of pH values 5.6, 6.4, 7.2, and 8.0 were prepared in a similar manner to that of the clots in which plasma concentration was varied. An initial mixture of desired pH was prepared by combining the constituents in the following proportions: five drops of plasma, two drops of buffer solution (either potassium acid phosphate or potassium acid phthalate), and five drops of embryonic extract, giving a standard plasma concentration of about 40 per cent. The pH of the buffer solution was determined by the Beckman pH meter, and, as a further check, a colorimetric pH determination of the clot mixture was made at the time of clotting. Here again, two drops of the prepared medium were quickly pipetted and spread on a series of glass cover slips before gelation occurred, and the clot was allowed to coagulate firmly before the tissue explant was placed on the surface. The cultures were then sealed and incubated.

Only by a continuous strong buffering action on the medium is it possible to maintain a constant pH in a tissue culture system. The plasma, the embryonic extract, and the growing tissues act as counterbuffers, tending to bring the system to equilibrium near pH 7.4. In the present experiments, no attempt was made to continuously buffer the clot and maintain a desired pH throughout incubation. It was observed that within eight hours the cultures had returned to pH 7.4, regardless of the initial pH at the time of clotting. Since the basic fine structure of the clot is set during the coagulation process, it was considered necessary for the purposes of the experiment to control pH only during that period.

Methods of measurement. Two types of data were obtained: 1) the rate of cell migration and 2) quantitative expressions of cell shape. Cells which are located on the cover slip surface or at the air-plasma interface exhibit shapes and migratory rates quite different from those of cells within the plasma clot. Since the problem under investigation is the reaction of cells to the structure of the fibrin framework, measurements were limited to only those cells definitely ascertained to be migrating inside the fibrin clot.

Camera lucida drawings of the living cultures were used in determining the rate of migration of cells in clots of varying texture (see Fig. 1). Because there is a period of lag before initial outgrowth which varies from culture to culture, the first data were recorded after 24 hours of incubation. At that time, a camera lucida drawing of each culture was made, showing the explant together with the outermost circle of cells. After 48 hours of incubation, a second image was superimposed on the original drawing, showing the new outermost circle of cells. The radial distance traversed by the farthest advanced cells between 24 and 48 hours of incubation was measured on the drawing, and the rate of migration was calculated in mm. per day by means of ocular micrometer measurements. Although individual cells could not be followed with certainty, it was assumed that by taking the average of a sufficiently large number of measurements, errors would be absorbed.

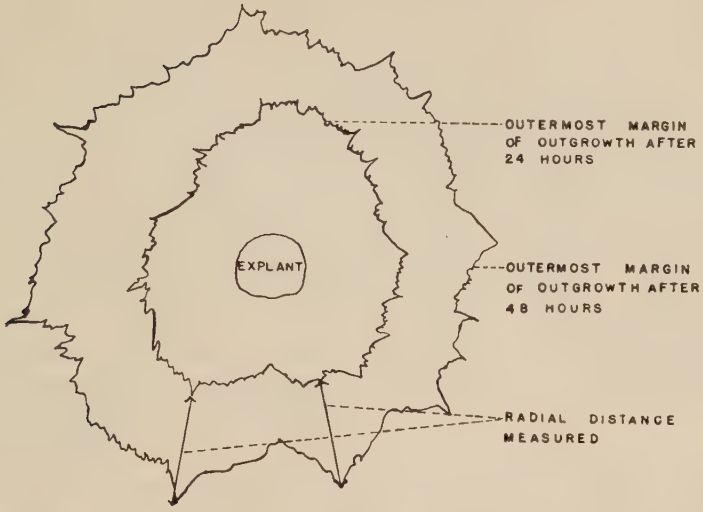

Fig. 1.

For the quantitative description of the morphological characteristics of cell populations growing in varying environments, the following methods were employed. After 48 hours of incubation, the clots, left intact on their original cover slips, were fixed in Ringer-formol solution and stained with Ehrlich's hematoxylin. Measurements of cell shape were taken from tracings of individual cell outlines projected by a carbon arc microprojector. In order to avoid the favoring of any particular cell type, the identification of the clot was masked, and all cells whose outlines were clearly defined and which lay within the clot were selected and drawn. Three types of data were taken: 1) each cell was classified as spindle-shaped or stellate, 2) the number of major pseudopodia of each cell was counted, and 3) the length and width of both the cell and the nucleus were recorded. These fundamental data were the basis of calculations made to express quantitatively the morphology of cell populations as indicated by 1) the proportion of spindle-shaped cells to stellate cells, 2) the frequency distribution of cells according to the number of major pseudopodia, and 3) the ratio of cell length to cell width, and the ratio of nuclear length to nuclear width.

RESULTS

Cell morphology. When measurements reflecting the cell shape of 2 417 fibrocytes were plotted against either the plasma concentration or the pH of the clot, smooth curves were obtained. This shows that the change in the form of the cell is a direct function of the change in the physical constitution of the medium. As the plasma concentration increases or as the pH decreases, there is an increase in the proportion of spindle-shaped cells, a decrease in

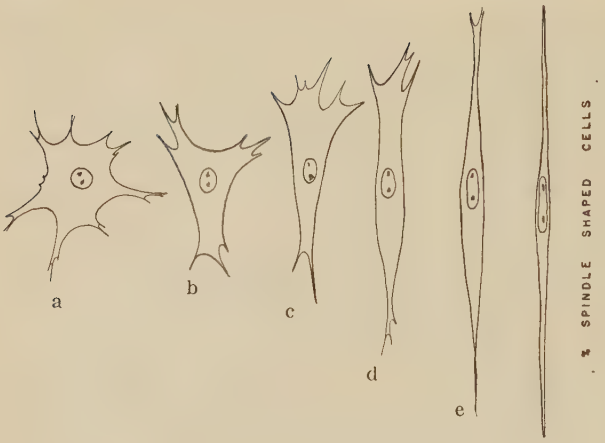

Fig. 2.

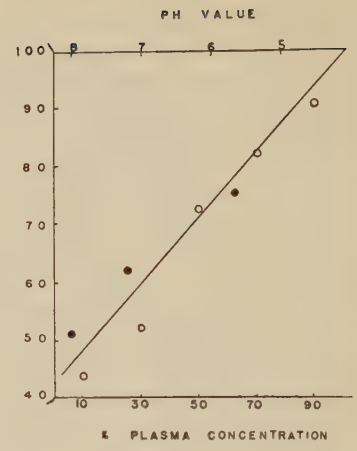

Fig. 3.

the number of pseudopodia per cell, and an increase in elongation of both the cell body and the nucleus.

For each parameter studied, the two curves obtained independently from the plasma variation series and the pH variation series could be fitted to a common curve by choosing suitable coordinate systems. Moreover, one and the same coordinate system was found to be appropriate for all parameters. Comparison of abscissa units shows that a medium of standard 40 per cent plasma at pH 8.0 corresponds to a medium of 6 per cent plasma at normal pH, while at the other end of the scale, a medium of 40 per cent plasma at pH 5.6 corresponds to a medium of 65 per cent plasma at normal pH. It is concluded from these results that the texture of the medium is dependent jointly upon the pH at the time of coagulation and upon the plasma concentration, and that cell shape is a function of a primary variable—the physical structure of the medium.

The plasma concentration series introduced differences of chemical composition due to the varying plasma-embryo extract ratios, along with the mentioned differences of texture. However, since in the pH series the same differences of texture were produced at constant compositions and the results were the same in both series, physical structure is revealed to be the relevant factor.

Proportion of spindle-shaped cells to stellate cells. The first and most general criterion used in analyzing cell morphology is the simple classification of cells as either stellate or spindle-shaped. A sample series of cell forms are shown in Fig. 2. Of this group, (a), (b), and (c) are classified as essentially

multipolar or stellate; (d), (e), and (f) are classified as essentially bipolar or spindle-shaped.

The graph in Fig. 3 shows the increase in the percentage of spindle-shaped cells and the accompanying decrease in the percentage of stellate cells as the plasma concentration of the medium increases or as the pH decreases. The curve obtained is a straight line whose upper limit passes through the ordinate of 100 per cent spindle-shaped cells at the theoretical plasma concentration of 100 per cent. The graph indicates a tendency toward spindle-shaped cell form in this standard strain of heart fibrocytes, for the lower limit of the curve approaches 43 per cent spindle-shape (rather than 0 per cent) at negligible plasma concentration values. In media of low plasma concentration, there are present a sizeable number of spindle-shaped cells, even though the majority are stellate cells.

Number of pseudopodia per cell. As a more precise determination of the amount of branching of the cell body, the number of major pseudopodia of each cell was counted. The histograms in Figs. 4 and 5 and the corresponding data in Table I illustrate the distribution of cells in clots of varying plasma concentration or pH when ranked according to the number of major pseudopodia per cell. There is a gradual transition from the situation where a large percentage of cells with four or more pseudopodia are found in clots of low plasma concentration or high pH value to the situation where a predominant percentage of cells with only two pseudopodia are found in clots of high plasma concentration and low pH value.

Thus, not only does the spindle-shaped to stellate ratio vary systematically with the clot constitution, but also the number of processes any given cell extends. In a clot of 90 per cent plasma, the great majority of spindle-shaped cells have only two branches and are strictly bipolar; while most spindle-shaped cells in a clot of 10 per cent plasma, although they are essentially bipolar, exhibit more branching and extend more than two processes. Similarly, a stellate cell representative of a 90 per cent plasma clot has fewer processes than a stellate cell representative of a 10 per cent plasma clot.

Elongation of cell and nucleus. Not only does the cell assume a spindle-shape and present fewer pseudopodia with increasing plasma concentration and decreasing pH, but, in addition, the elongation of both the cell body and the nucleus becomes increasingly marked. This effect is clearly illustrated in Fig. 6, where the index of elongation of both the cell (E_c) and the nucleus (E_n), expressed quantitatively as the ratio of cell length to cell width and the ratio of nuclear length to nuclear width, are plotted against the coincident scales of plasma concentration and pH values. As shown by the

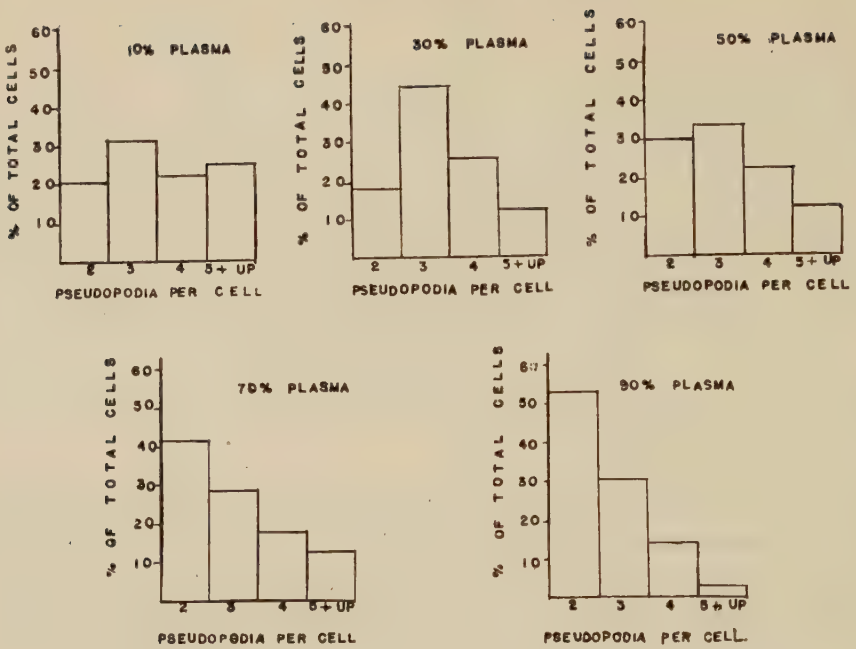
10% PLASMA
30% PLASMA
50% PLASMA
70% PLASMA
90% PLASMA
% OF TOTAL CELLS
PSEUDOPODIA PER CELL
2
3
4
5+ UP

Fig. 4.

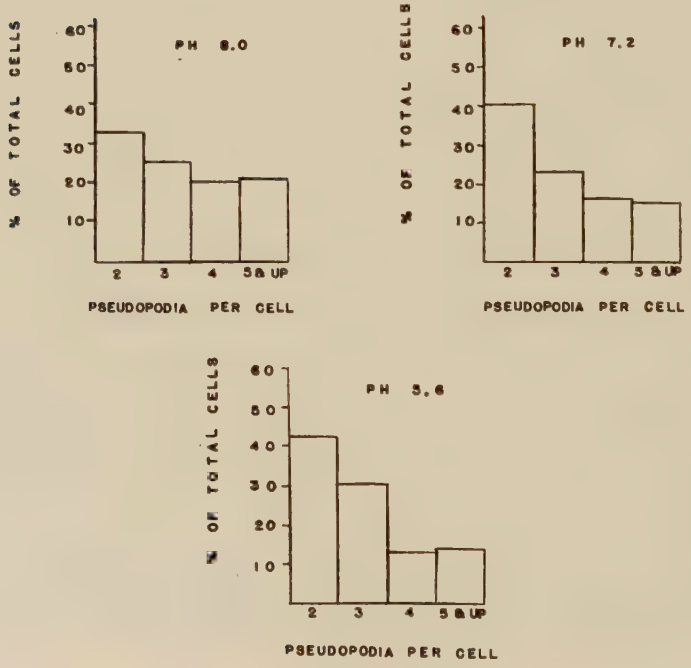
PH 8.0
PH 7.2
PH 5.6
% OF TOTAL CELLS
PSEUDOPODIA PER CELL
2
3
4
5 & UP

Fig. 5.

TABLE I

Distribution of Cells According to Number of Pseudopodia.

Constitution of Clot	Percentage of Cells				No. of Cells Measured	No. of Clots Measured
	2 Pseudo-podia	3 Pseudo-podia	4 Pseudo-podia	5+ Pseudo-podia		
Plasma Concentration Series.						
10 per cent Plasma....	20.2	31.1	23.7	25.0	405	8
30 » » »	18.7	44.5	25.7	12.0	237	5
50 » » »	29.8	33.2	24.8	12.2	383	8
70 » » »	41.8	28.4	17.6	12.2	222	7
90 » » »	53.1	30.3	13.8	2.8	145	8
Total	–	–	–	–	1 392	36
pH Series.						
pH 8.0	32.7	25.8	20.6	20.9	349	7
pH 7.2	40.4	27.6	16.7	15.3	425	7
pH 5.6	42.3	30.2	13.5	14.3	251	5
Total	–	–	–	–	1 025	19

graph in Fig. 6 curves of non-linear character are obtained. The fact that the cell achieves a greater elongation than the nucleus is revealed by a comparison of the two curves. Furthermore, there is a constant relationship between the index of elongation of the cell and that of the nucleus, the ratio $E_c : E_n$ averaging a constant value of 3.4.

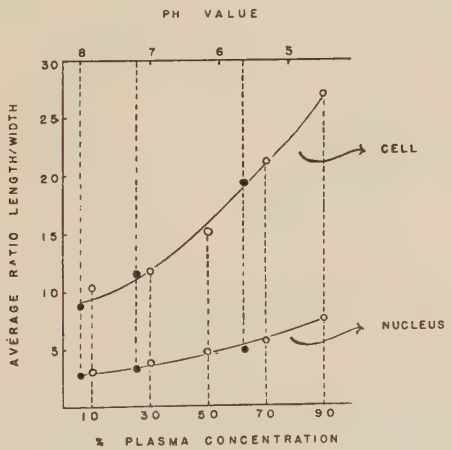

Fig. 6.

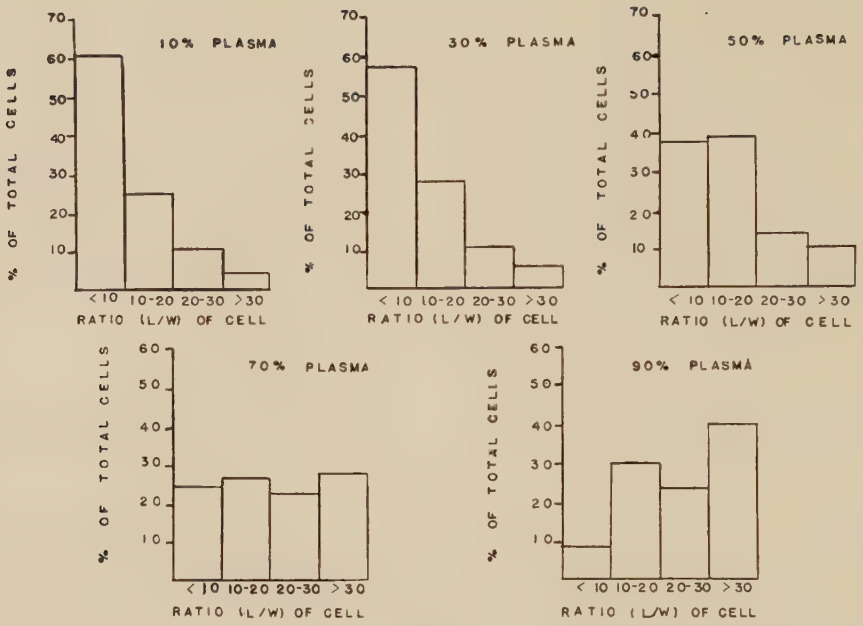
10% PLASMA
30% PLASMA
50% PLASMA
70% PLASMA
90% PLASMA
% OF TOTAL CELLS
< 10 10-20 20-30 > 30
RATIO (L/W) OF CELL

Fig. 7.

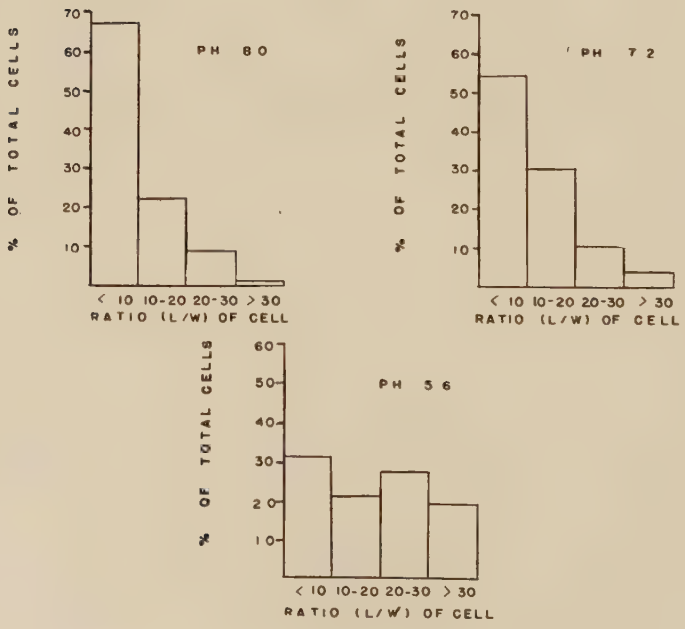
PH 8 0
PH 7 2
PH 5 6
% OF TOTAL CELLS
< 10 10-20 20-30 > 30
RATIO (L/W) OF CELL

Fig. 8.

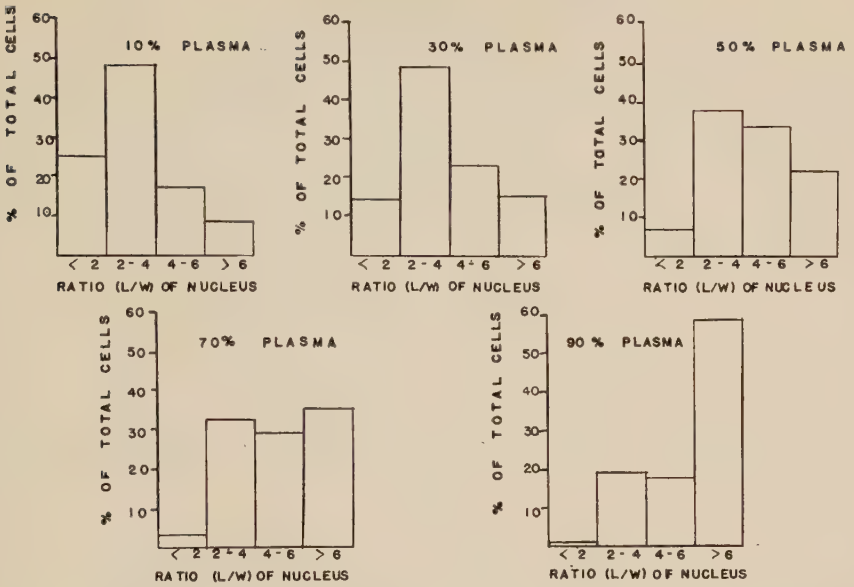

Fig. 9.

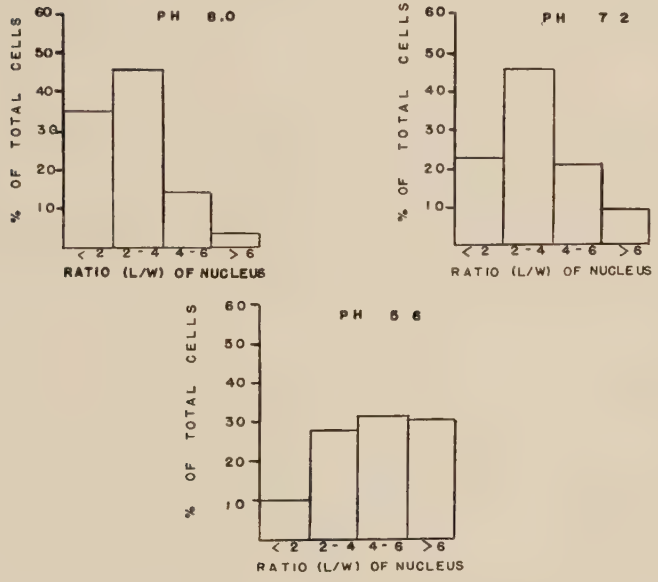

Fig. 10.

The histograms in Figs. 7–10 and the corresponding data in Tables II and III illustrate the distribution of individual cells in the culture population when ranked according to the indices of cell and nuclear elongation. As the

TABLE II

Distribution of Cells According to Degree of Cell Elongation.

Constitution of Clot	Percentage of Cells Ranked According to Value of Ratio $\frac{L}{W}$ of Cell				No. of Cells Measured	No. of Clots Measured
	Under 10	10–20	20–30	30+		
Plasma Concentration Series.						
10 per cent Plasma....	61.2	25.2	10.6	4.4	405	8
30 » » »	57.0	27.5	10.6	5.4	237	5
50 » » »	37.1	38.7	13.8	10.5	383	8
70 » » »	23.9	26.6	22.5	27.1	222	7
90 » » »	8.3	29.0	23.4	39.5	145	8
Total	–	–	–	–	1 392	36
pH Series.						
pH 8.0	67.3	22.4	8.9	1.4	349	7
pH 7.2	54.4	30.8	10.8	4.0	425	7
pH 5.6	31.5	21.9	27.5	19.1	251	5
Total	–	–	–	–	1 025	19

TABLE III

Distribution of Cells According to Degree of Nuclear Elongation.

Constitution of Clot	Percentage of Cells Ranked According to Value of Ratio $\frac{L}{W}$ of Nucleus				No. of Cells Measured	No. of Clots Measured
	Under 2	2–4	4–6	Over 6		
Plasma Concentration Series.						
10 per cent Plasma....	25.1	48.6	17.5	8.5	405	8
30 » » »	14.8	48.6	23.7	13.0	237	5
50 » » »	7.0	37.6	33.2	22.0	383	8
70 » » »	2.8	32.5	29.3	35.7	222	7
90 » » »	2.1	19.3	18.7	59.9	145	8
Total	–	–	–	–	1 392	36
pH Series.						
pH 8.0	34.9	46.7	14.6	3.7	349	7
pH 7.2	23.0	46.4	21.2	9.4	425	7
pH 5.6	10.0	27.9	31.5	30.6	251	5
Total	–	–	–	–	1 025	19

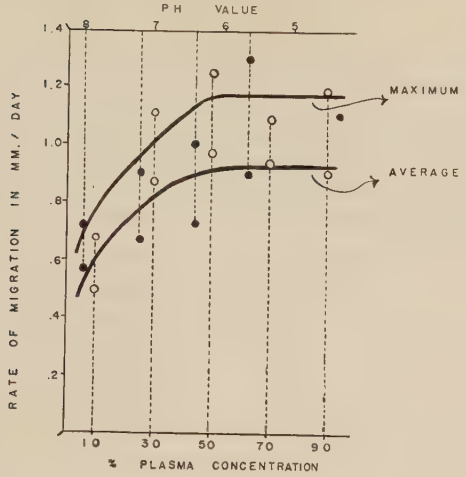

Fig. 11.

plasma concentration increases or as the pH decreases, there is a steady increase in the frequencies of those classes of higher index values. These graphs clearly show the systematic changes in the composition of the cell population according to their physical environment. Where it becomes increasingly difficult to recognize differences in the morphology of cells by qualitative appraisal, these quantitative expressions of cell shape obtained from actual cell and nuclear dimensions define the differences.

Cell migration. In measuring the rate of cell migration within clots of different physical constitution, it was found that cells in medium of high plasma content or low pH value travel farther within a 24 hour period than cells in a medium of low plasma content or high pH value. The actual values of migration rates of 2 342 cells in clots of different physical constitution are shown in Fig. 11 and Table IV.

In the plasma concentration series the average rate of migration increases from 0.50 mm. per day for cells in a clot of 10 per cent plasma to 0.97 mm. per day in a clot of 50 per cent plasma. However, with further increase of plasma concentration, the rate of migration no longer increases. Rather, a plateau is reached and the rate tends to remain constant thereafter. The same type of curve is obtained when the maximum rates are plotted. The maximum value of approximately one mm. per day is in good agreement with the rate of migration recorded for Schwann cells travelling along glass fibers in tissue culture clots (23).

In examining the data obtained from the experimental series in which pH

TABLE IV

Rate of Migration.

Constitution of Clot	Average Rate of Migration (mm/day)	Maximum Rate of Migration (mm/day)	No. of Cells Measured	No. of Clots Measured
Plasma Concentration Series.				
10 per cent Plasma	0.498	0.679	183	5
30 » » »	0.875	1.110	215	5
50 » » »	0.973	1.254	402	9
70 » » »	0.945	1.093	399	9
90 » » »	0.910	1.193	383	8
Total	–	–	1 582	36
pH Series.				
pH 8.0	0.571	0.725	190	4
pH 7.2	0.678	0.904	210	4
pH 6.4	0.717	0.981	180	4
pH 5.6	0.914	1.303	180	3
Total	–	–	760	15

was altered at the time of clotting, it is found that as the pH decreases from 8.0 to 5.6, there is a steady increase in the rate of migration, just as in the corresponding plasma concentration gradation from 6 per cent plasma to 65 per cent plasma, although this increase is less rapid. It was not possible to test whether an eventual plateau is reached, because plasma will not coagulate at pH values below 5.5. However, it will be noted that the rate of migration obtained at pH 5.6 does not differ appreciably from the rate at the plateau level in the plasma concentration series.

DISCUSSION

The results described in the foregoing (a) prove the dependence of cell shape and cell movement (for a given cell type) upon the physical structure of the medium, and (b) express this dependence in quantitative form. Beyond this it has been possible to derive the nature and direction of the observed quantitative variability from a consideration of the properties of the clot, particularly the texture of its fibrin framework, and of the reactions of the

cells extending and moving along this network as first described by Harrison (11) and later elaborated by Weiss (19, 20, 23) and others. This derivation has been given in a previous paper (25) to which the reader is referred for a detailed explanation. Briefly, these are the salient points.

The clotting of plasma involves the progressive aggregation of filiform fibrin units into longer and thicker strands (1, 8, 16). The ultimate degree of bundling, hence, the average coarseness of the fibrin network increases with rising fibrin concentration as well as with diminishing pH. Increasing aggregation also yields a greater range of fiber sizes.

Cell shape is essentially an expression of the momentary state of locomotor equilibrium, as it is determined by the number, distribution and orientation of the various mobile cell processes (pseudopodia). As explained in our previous article, this pseudopodial pattern is a function of (a) the frequency of coarse fibrin filaments; (b) the range of the size spectrum of fibrin fibers; (c) the surface constitution of the cell; and (d) hydrodynamic and elastic competition among multiple processes. By taking these variables into account, the various morphological adaptations described in this paper (average number of processes and their degree of antipodal distribution from multipolarity to strict bipolarity) can be satisfactorily explained. The mechanism by which nuclear shape is made to conform to the shape of the cell body has not yet been ascertained.

The rate of locomotor progress of a fibrocyte increases with the mounting coarseness of the fibrin framework but reaches a maximum at 50 per cent plasma concentration. Further increase in the coarseness of the clot is not accompanied by higher locomotor rates. Evidently, the lower rates of migration in clots of relatively fine texture are due to the more frequent delays in forward progress as multiple pseudopodia are extended and withdrawn in directions other than those in the main line of advance. In a network of coarse fibrin bundles amid finer strands, a cell extends preferentially along a thick bundle, assumes a bipolar form, and advances steadily at a maximum rate. Once in this condition, augmented abundance of thick fibrin strands can have no further effect on locomotor behavior although the stretching of the cell body to slenderer proportions continues. This fact imposes the exercise of great caution in using the rate of expansion of the contour of a culture as an index of "growth" in general, or for comparative purposes, in the assaying of "growth factors." As pointed out previously (25), one must distinguish between effects which these factors may have on cell morphology and locomotion directly and those produced by a primary change in the medium or in the reaction of the cells to it.

SUMMARY

1. Standard tissue cultures of chick heart fibrocytes were grown in plasma clots whose physical constitution was systematically altered, either by varying the pH at the time of clotting through a range of pH 8.0-5.6, or by varying the plasma concentration through a range of 10–90 per cent plasma. Other conditions were kept constant.

2. Measurements of 4759 cells plotted against plasma concentration or pH produced smooth curves, showing transitional responses.

3. With increasing plasma concentration and decreasing pH, there is a corresponding increase in the percentage of spindle-shaped cells, a decrease in the number of pseudopodia per cell, an increase in the ratio of cell length to cell width, and an increase in the ratio of nuclear length to nuclear width.

4. The rate of cell migration increases as the plasma concentration increases or as the pH decreases, but reaches a constant level above 50 per cent plasma concentration.

5. An interpretation of the results in terms of the properties of the fibrin framework and of the reactions of the cells to it has been given on a previous occasion (25).

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SEPARATION OF MITOCHONDRIA INTO TWO MORPHOLOGICALLY AND BIOCHEMICALLY DISTINCT TYPES

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The mitochondrial pellet obtained from rat liver by the cell fractionation technic of Hogeboom, Schneider, and Pallade (5) consists of two layers: a lower, more densely packed, tan layer, and an upper, poorly sedimented, pinkish, fluffy layer (Fig. 1). Several authors (8, 10, 13) have noted the occurrence of this layer and have emphasized that it should be separated from the more densely packed particles, which are generally agreed to be mitochondria. Schneider and Hogeboom (13) and Muntwyler *et al.* (8) have combined the fluffy layer with the microsome fraction.

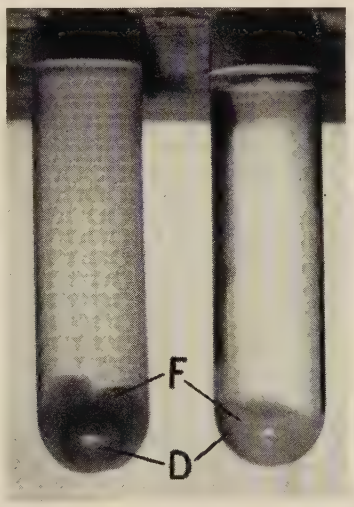

Fig. 1. Mitochondrial pellet, showing the dense layer (D) and the fluffy layer (F). The tube on the right was allowed to stand upright for some time after sedimentation; the dense layer has not moved, but the fluffy layer has settled to the bottom of the tube.

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In the present work, the fluffy layer material of rat liver has been isolated by repeated washings and resedimentations. The isolated fluffy layer material has been compared with the mitochondria of the more densely packed layer and with the microsomes, by phase contrast and electron microscopy and by various analytical and enzymatic technics. A fluffy layer fraction, apparently corresponding to that of rat liver, has been separated from the pancreas and submaxillary gland of the rat, the human adrenal, and three tumors.

MATERIALS AND METHODS

Male albino rats of the Sprague-Dawley strain were used for the fractionations of liver, pancreas, and submaxillary gland. They were fed Friskies dog pellets supplemented with a whole grain diet, and water, *ad libitum*. They were not fasted prior to use. The livers of two to four rats, and the pancreas and submaxillary tissue from ten to thirteen rats were pooled for each fractionation.

The human adrenal[1] was removed at surgery from a 35-year old man. It was normal in appearance, but was removed with the adjacent kidney, which bore the hypernephroma described below. The whole surgical specimen was chilled immediately, and the tissues were kept chilled throughout the fractionation procedure. The entire adrenal gland was fractionated, but since in the human the cortex comprises the major part of this gland, the values obtained in this experiment are probably characteristic of the cortical tissue. The hypernephroma was a large tumor with scattered foci of necrosis; only grossly non-necrotic tissue was used for fractionation. A transplantable adrenal tumor of the mouse[2] was also fractionated. The third tumor was a rapidly growing fibrosarcoma of the thigh removed at surgery from a 50-year old woman.[1] Although it was a large tumor, very little evidence of necrosis was present; only grossly non-necrotic tissue was used for fractionation.

Experimental animals were killed with ether; the organ to be studied was removed quickly and chilled, and all further operations were carried out at 0–5° C. The livers were perfused with ice-cold isotonic sucrose; the other organs were not perfused. The tissues were minced by pressing through a lucite sieve to remove as much as possible of the connective tissue, and the tissue mince was then homogenized in a lucite Potter-Elvehjem apparatus in a concentration of 12 per cent by weight in 0.88 M sucrose.

[1] The human tumors were obtained through the courtesy of Dr. Walter Jaeschke, surgical pathologist of the State of Wisconsin General Hospital.

[2] The transplantable adrenal tumor was orginally obtained from Dr. G. W. Wolley, of the Sloan-Kettering Institute for Cancer Research, New York.

Figs. 2–13. Electron micrographs of isolated cytoplasmic particles prepared in 0.88 M sucrose, fixed in OsO_4, and shadowed with uranium. (RCA Universal type electron microscope.) Original magnification in electron microscope, Figs. 2–12, ×2 870; Fig. 13, ×7 610. Magnification as shown: Figs. 2–7, ×4 060; Figs. 8 and 9, ×5 070; Figs. 10–12, ×4 560; Fig. 13, ×12 350.

Figs. 2, 3. Large mitochondria from rat liver, washed four times.

Figs. 4, 5, 6. Small mitochondria from the fluffy layer of rat liver, washed four times.

Fig. 7. Microsomes from rat liver.

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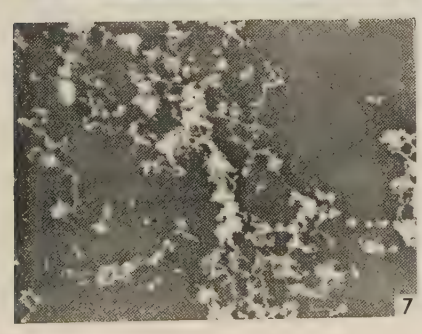
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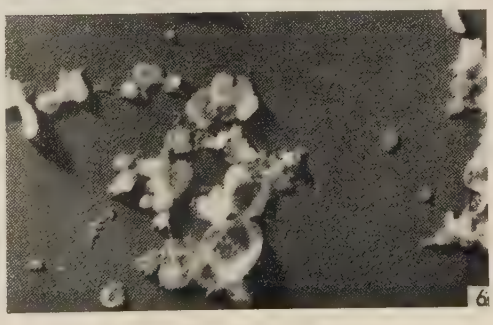
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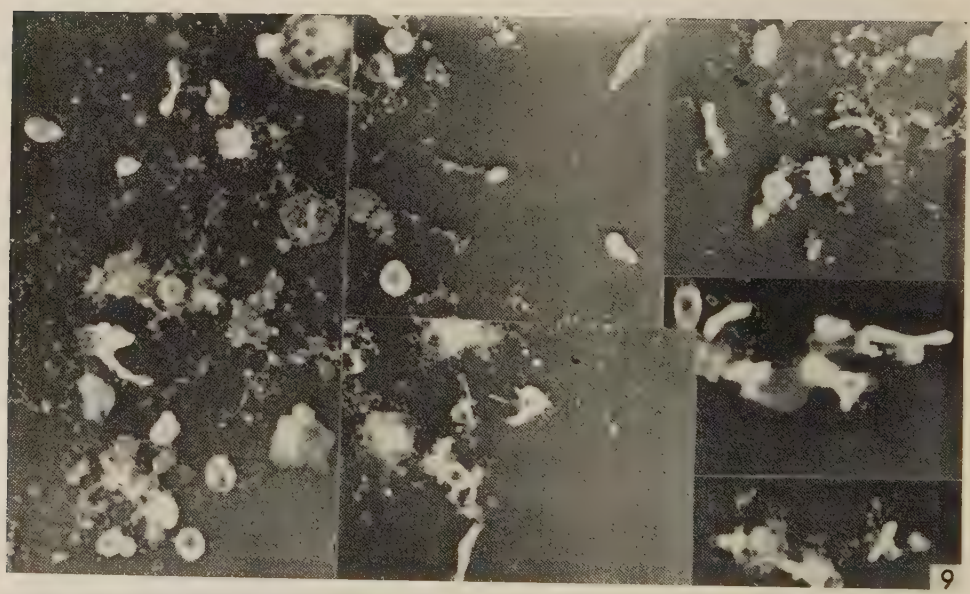
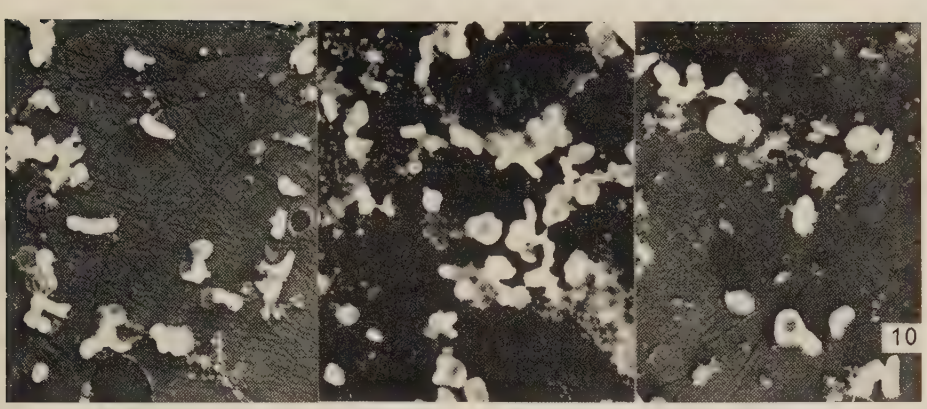


Fig. 8. Large mitochondria from human adrenal. The "doughnut" or "tennis-racket" form seems to be characteristic of the large and small mitochondria of this tissue. Washed once.
Fig. 9. Small mitchondria from human adrenal, washed once.

Fig. 10. Large mitochondria from the human hypernephroma, washed once.
Fig. 11. Small mitochondria from human hypernephroma, washed once.
Figs. 12, 13. Microsomes from human hypernephroma.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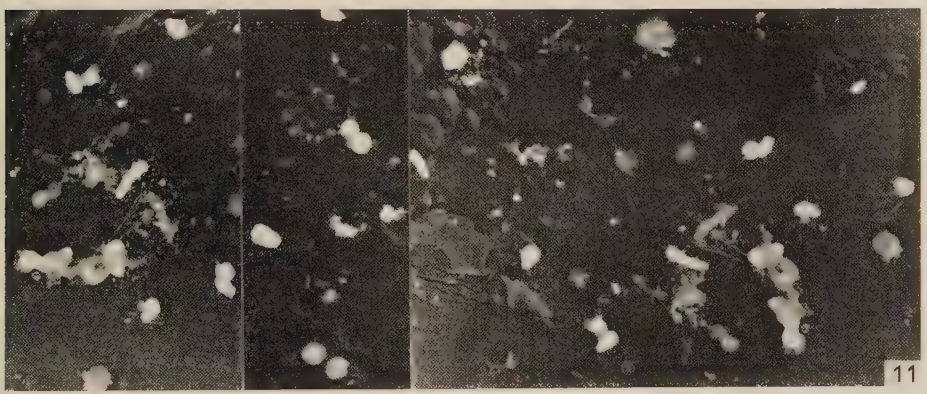
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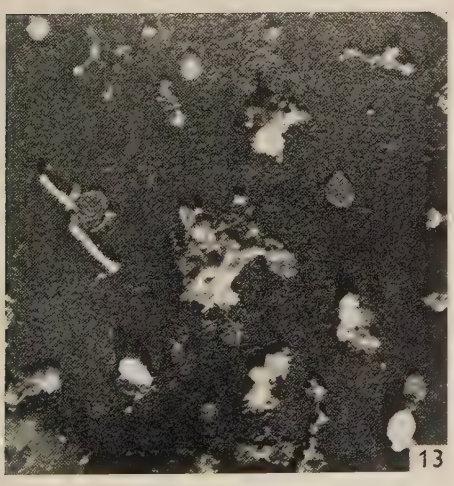
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Centrifugal fractionation. The nuclei were sedimented at 600 × g for 10 minutes, and discarded. The mitochondrial pellet was obtained by centrifuging at 18 000 × g for 10 minutes. The supernate was removed and the mitochondrial pellet (including the fluffy layer) was resuspended in about 10 volumes of the sucrose medium and resedimented at 18 000 × g for 10 minutes. Once more the supernate was removed. The fluffy layer overlying the dense pellet (Fig. 1) was then removed by pipette, and the surface of the dense pellet was rinsed four times with about one ml of the sucrose medium, the rinses being added to the fluffy layer suspension. The dense pellet and the fluffy layer material were then suspended separately in about 10 volumes of the sucrose medium, and were recentrifuged at 18 000 × g for 10 minutes. The fluffy layers from both sediments were combined, and the dense pellets similarly combined, and the washing procedure repeated. In most of the work, washing and recombination of the two types of sediment were repeated four times. For the final separation, the top of the fluffy layer and the bottom of the dense pellet were collected. Sedimentation of the mitochondria and fluffy layer was carried out in an aluminium head which supported the tubes at an angle of 22° from vertical: the gravitational force was 12 600 × g at the top, 18 000 × g at the center, and 23 400 × g at the bottom of the tubes. An International centrifuge, Model PR-1, with high speed attachment, was used. The microsome fraction was obtained by centrifuging the first two mitochondrial supernates together at 120 000 × g for 36 minutes.

In the fractionations of tissues other than rat liver, the two mitochondrial subfractions were separated by a less elaborate procedure. After resuspension and resedimentation of the initial mitochondrial pellet, the surface of the dense pellet was rinsed as in the liver experiments and the washings were added to the fluffy layer fraction. The sub-fractions were not washed further.

Analytical methods. The particulate fractions were made up to suitable volumes in the homogenizing medium, and aliquots were taken for microscopic examination, chemical analyses, and enzyme study. Chemical analyses included determination of protein-nitrogen by a Nesslerization procedure (15), nucleic acids by Schneider's method (12), and lipid phosphorus by two methods: Schneider's method (12) was used in the first case, and in the second the tissue fraction was pipetted directly into hot alcohol-ether, and the lipid extract so obtained was evaporated to dryness, re-extracted with petroleum ether, and evaporated to dryness again. The residue was digested and analyzed for phosphorus as in Schneider's method. Enzyme studies included determination of succinoxidase activity and succinic dehydrogenase activity (14), and acid and alkaline phosphatase activities. The substrate for the phosphatase determinations was β-glycerol phosphate (0.012 M) and the reactions were run at pH 5.3 (0.005 M phthalate buffer) and pH 9.1 (0.005 M KOH-glycine buffer), at 30° C, for 30 minutes. The tissue samples contained approximately 1 mg of protein-nitrogen. Under these conditions the amount of inorganic phosphorus liberated was proportional to the amount of tissue sample used, and in each case less than 3 per cent of the substrate was hydrolyzed. The phosphate liberated was determined by the method of Fiske and SubbaRow.

Combination of the three particulates with Janus Green B was also investigated. The dye was added in a final concentration of about 1 : 300 000 to a suspension of the particles in a centrifuge tube. The sediment was obtained by centrifugation at 18 000 × g for 10 minutes, and the tube with its contents was incubated at 37° C for 30 to

60 minutes.[1] This procedure was carried out under air or nitrogen, and with or without the addition of succinate to the medium before centrifuging, in a final concentration of about M/30. The concentration of the dye in the pellet, and the reduction of the dye to the red-colored safranin form were observed qualitatively.

Microscopy. The particulates were prepared for electron microscopy by fixation with OsO_4 as soon as possible after completion of the fractionation procedure. A few drops of 2 per cent OsO_4 were added directly to about one ml of a suspension of the particles in the sucrose medium. The suspension was then smeared on a formvar-covered microscope slide, dried, and rinsed in distilled water to remove the heavy deposit of sucrose. The formvar film was then shadowed with uranium and transferred to the customary screens for observation with the electron microscope.[2]

Isotopic labeling. In order to test the possibility of cross-contamination of fractions, the following experiment was carried out. Fluffy layer and microsome particles labeled with P^{32} were obtained from normal rats injected about 15 hours previously with 100 microcuries of $P^{32}O_4$ per 250 g rat. The labeled particles were added separately to the two halves of a liver mince prepared from unlabeled livers, and each of the two samples of mince was homogenized and separated by the above technic into the two mitochondrial sub-fractions and the microsome fraction. The distribution of the P^{32} in the fractions was determined in terms of total counts present in each fraction, and as specific activity of P^{32} in the pentosenucleic acid (PNA) of the particulate fractions.

RESULTS

Microscopy. Under the phase contrast microscope the two mitochondrial subfractions of rat liver were seen to be composed of particles similar in shape but differing in size. The particles were mainly short rods, although many vesicles of the sort described by Harman (4) as characteristic of mitochondria were present in both fractions. The particles of the fluffy layer were distinctly smaller than those of the dense layer, but no quantitative estimate of size differences could be made at the magnification possible with the light microscope. The fluffy layer particles were more highly refractile, as shown by their darker appearance when viewed through a dark phase contrast lens system. Very few particles were seen in the microsome fraction, seldom more than two or three per high power field.

At the higher magnification possible with the electron microscope, the particles could be measured (Figs. 2–7), and it was found that those of the fluffy layer averaged about one-half the size of the more densely packed

[1] This method of studying the concentration and reduction of Janus Green B by isolated particulates was given to us by Dr. R. O. Recknagel, formerly of the McArdle Memorial Laboratory and presently at the University of Utah. We are grateful to him for suggestions concerning its use in the present work.

[2] The electron microscopy was done in the electron microscope laboratory of the University of Wisconsin. We wish to thank Dr. Paul Kaesberg for his advice and help.

TABLE I

Relative proportions of PNA, lipid-phosphorus, succinoxidase, and phosphatase in the cytoplasmic particulates of rat liver.

	mg PNA / mg Protein-N	mg Lipid-P / mg Protein-N	Succinoxidase[1] Protein-N	Phosphatase (μgP/mg Protein-N)	
				Acid	Alkaline
Large mitochondria	.06 (.02–.16)	.172[a], .183[b]	100	3.70, 2.39	1.11, 0.92
Small mitochondria	.52 (.39–.69)	.284[a], .342[b]	76 (52–88)	3.02, 3.04	2.98, 3.13
Microsomes	.62 (.45–.83)	.455[a], .404[b]	16 (15–18)	3.34, 2.78	1.41, 1.31

[a] By Schneider's method (12).
[b] By direct lipid extraction (see text).

particles of the mitochondrial fraction in each dimension. A considerable range of sizes was present in each fraction, but the little overlapping which was noted could be accounted for by slight contamination of one fraction with another. Little contamination with microsomes was evident in either of the mitochondrial preparations, and was no greater in the fluffy layer than in the more densely packed mitochondrial layer. The microsomes also contained some rods and vesicles (Fig. 7), similar in shape to those observed in the mitochondria, but very much below the size range of the small mitochondria and below the limit of resolution of the light microscope. When the fluffy layer and microsome fractions were suspended three hours in distilled water and then fixed with OsO_4, almost all the particles in both preparations were vesiculated. The vesicles of the fluffy layer fraction collapsed on drying and appeared flat, while those of the microsome fraction were somewhat dome-shaped. Both fractions contained a few small, dense, non-vesiculated spheres, which were readily distinguished from the vesicles. A few of these dense particles were seen under the phase contrast microscope in fixed fluffy layer suspensions; it was estimated that they constitute less than 10 per cent of the material in this fraction.

Analytical results. The results of the chemical and enzyme determinations on the particulates of rat liver are presented in Table I. The values given are based on protein-nitrogen. For reasons given in the discussion, the particles of the dense layer have been called large mitochondria and those of the fluffy layer have been called small mitochondria.

The small mitochondria have an average succinoxidase activity 76 per

[1] Per cent of activity of most active fraction. Range of values from 6 fractionations given in parentheses.

TABLE II

Proportions of cytoplasmic particulates in various tissues.

	Large mitochondria		Small mitochondria		Microsomes	
	Protein-N	PNA/N	Protein-N	PNA/N	Protein-N	PNA/N
Rat liver (1)	2.57	.04	4.58	.40	4.03	.81
(2)	2.34	.07	4.38	.41	3.82	.79
Human adrenal	0.88	.16	0.45	.20	1.85	.49
Rat pancreas (1)	0.52	.12	1.18	.35	6.86	1.5
(2)	1.05	.04	1.11	.36	7.72	1.6
Rat submaxiliary gland (1)	0.13	.38	0.92	.25	3.82	1.3
(2)	0.14	.43	0.94	.17	3.62	1.4
Tumors, mouse adrenal...	0.54	.11	0.76	.33	2.12	1.1
human fibrosacroma ...	0.18	.08	1.03	.40	1.77	.61
human hypernephroma	0.08	.25	0.13	.23	0.38	.29

Protein-nitrogen as mgm per g of tissue, wet weight.
These fractions, including those of rat liver, were all obtained by the simplified technic described in the text.

cent of that of the large mitochondria, but they have a PNA to protein-nitrogen ratio 84 per cent of that of the microsomes on the average. In three of the six fractionations reported in Table I, the PNA to protein-nitrogen ratio of the small mitochondria was 101, 109 and 94 per cent of that of the microsomes. The PNA to protein-nitrogen ratio of the large mitochondria is extremely low, in contrast to those of the other two cytoplasmic particulates. The relative distribution of succinic dehydrogenase activity was similar to that of the succinoxidase. No measurable desoxypentosenucleic acid was found in these particulate fractions.

All three fractions were found to concentrate Janus Green B in the pellet obtained by centrifugation. However, only the denser mitochondrial pellet reduced the dye to the red-colored safranin form on incubation at 37° C under aerobic conditions without the addition of succinate, in agreement with the observations of Potter *et al.* (10). On incubation of the pellets from the succinate medium under anaerobic conditions, all three fractions reduced Janus Green B.

In the course of fractionation of rat pancreas and submaxillary gland, human adrenal, two human tumors and a mouse adrenal tumor, the mitochondrial fraction was separated into two sub-fractions by means of the simplified procedure already described. The results of the protein-nitrogen and pentose nucleic acid analyses are given in Table II. It is evident that

TABLE III

Distribution of P^{32} in PNA of fractions isolated from rat liver mince to which labeled microsomes[1] were added.

	Total counts in PNA (c/m)	Specific activity (c/m/μg PNA)
Large mitochondria	340	10.3
Small mitochondria	600	1.59
Microsomes	16 600	7.70
Nuclear fraction (unwashed)	11 400	
Final supernate	20 600	
Fluffy layer sediment (mixture of large and small mitochondria)	690	
Fluffy layer washes	9 380	
Total recovery	59 600	
Total counts added	63 800	
Total counts associated with fluffy layer (large and small mitochondria, fluffy layer sediment)	1 630	

even without special washing procedures, a large mitochondria fraction with a very low ratio of PNA to protein-nitrogen can be obtained from many tissues. However, it is also evident from the electon micrographs (Figs. 8–13) that this simplified procedure does not achieve complete separation of mitochondria, fluffy layer particles, and microsomes. Although not shown in electron micrographs here, this is true of rat liver also. On the other hand, it is clear that the more extensive washing procedure used for rat liver effectively separates the fluffy layer particles from both the large mitochondria and the microsomes (Figs. 2–7).

Isotopic labeling. Table III gives the distribution of P^{32} in the PNA of fractions separated from a rat liver mince after addition of labeled microsomes, and Table IV gives the corresponding data for fractions from a mince to which labeled fluffy layer was added. In both these fractionations, as in all our attempts to obtain pure cytoplasmic fractions, recovery has been sacrificed for the sake of purity. In both experiments, the nuclear fraction was not washed, and would be expected to contain a considerable amount of fluffy layer and microsomes.

[1] The specific activity of the PNA in the microsome fraction added was 35.9 c/m/μg PNA.

TABLE IV

Distribution of P^{32} in PNA of fractions isolated from rat liver mince to which labeled fluffy layer[1] was added.

	Total counts in PNA (c/m)	Specific activity (c/m/μg PNA)
Large mitochondria	490	23.8
Small mitochondria	2 300	6.43
Microsomes	5 870	4.83
Nuclear fraction (unwashed)	11 000	
Final supernate	4 850	
Fluffy layer sediment (mixture of large and small mitchondria)	8 860	
Fluffy layer washes	16 100	
Total recovery	49 470	
Total counts added	42 000	
Total counts associated with fluffy layer (large and small mitochondria, fluffy layer sediment, and fluffy layer washes)	27 750	

When the liver mince containing labeled microsomes was fractionated, 26 per cent of the added counts were recovered in the purified microsome fraction. At most 2.5 per cent of the added counts were associated with the fluffy layer. The counts in the fluffy layer washes are believed to be associated with microsomes which are washed out during the purification of the fluffy layer. Of the remaining 70 per cent, 18 per cent were found in the unwashed nuclear fraction, and 32 per cent in the final supernate.

When the liver mince containing labeled fluffy layer was fractionated, a considerable amount of radioactivity was found in the fluffy layer washes, the microsomes, and the final supernate. Even so, about 60 per cent of the added counts were associated with the fluffy layer. Since some of the fluffy layer particles as well as microsomes are lost in the fluffy layer washes, it is felt that in this case the counts in the washes represent counts associated with the fluffy layer.

It should be emphasized that the labeled fractions were "older" than those usually studied, since they were carried through a second fractionation procedure after their initial preparation. Under these circumstances, some breakdown of particles with release of P^{32} would be expected.

[1] The specific activity of the PNA in the fluffy layer fraction added was 29.2 c/m/μg PNA.

DISCUSSION

The existence of more than one kind of mitochondria has been considered (1, 3, 7, 9). Randavel-Vendrely (11), using cytochemical technics, demonstrated two kinds of mitochondria, one containing PNA and one without PNA.

Our washed fluffy layer material is similar to both mitochondria and microsomes (Table I), suggesting that this material might be simply a mixture of mitochondria and microsomes. However, this conclusion is untenable for a number of reasons. The fluffy layer cannot be 76 per cent mitochondria (on the basis of succinoxidase activity) and at the same time be 84 per cent microsomes (on the basis of the PNA to protein-nitrogen ratio). The alkaline phosphatase activity of this material is approximately twice that of the mitochondria or microsome fractions. The electron micrographs (Figs. 4–6) indicate that this material consists almost exclusively of one type of particle and that the PNA of this material is not due to freely suspended microsomes. The isotopic labeling experiments confirm this and also indicate that the PNA of this material is not due to clumped or adsorbed microsomes, since only about 2.5 per cent of the counts of the added microsome fraction were recovered in association with the fluffy layer. Moreover, the specific activity of the PNA of this fraction was about one-fifth of that of the microsomes, showing that this fraction contained PNA which diluted the PNA which came from the microsome fraction. In recent studies in which the uptake *in vivo* of $P^{32}O_4$ and glycine-2-C^{14} has been investigated, we have found that the specific activity of the PNA of the fluffy layer has invariably been considerably lower than that of the microsomes; the difference is too great to be due to dilution by the very small amount of PNA which is found in the mitochondria of the dense layer (to be published elsewhere).

Therefore, the washed fluffy layer material consists almost exclusively of a distinct class of particles. They are smaller than the mitochondria of the dense layer, but their similarity to the larger particles in shape, osmotic behavior, and succinoxidase activity suggests that they are mitochondrial in nature. Therefore we have called them "small mitochondria". However, it must be emphasized that although the particles in each of these mitochondrial sub-fractions show substantially uniform sedimentation and osmotic behavior, there is some variation in the shape and considerable variation in the size of the particles. This non-uniformity suggests the possibility of a functional heterogeneity which would make further fractionation desirable.

Some of the particles of the microsome fraction prepared in hypertonic sucrose closely resemble mitochondria in rod-like shape and in vesicle formation (Figs. 7, 12, 13), although they are very much smaller than even the small mitochondria. The succinoxidase activity found in this fraction (about 15 per cent of that of the large mitochondria) may be associated with such particles. These particles thus might constitute another class of mitochondria, of extremely small size. Dalton *et al.* (2) have also demonstrated the existence of mitochondria-like particles which are below the limit of resolution of the light microscope.

The ease with which the microsome fraction as well as the mitochondrial fractions can be made to reduce Janus Green B after concentrating it casts doubt on the specificity of Janus Green B as a criterion for distinguishing mitochondria from other cytoplasmic particulates. Our results and conclusions in this connection are in substantial agreement with those of Lazarow and Cooperstein (6).

The proportion of the total tissue PNA recovered in the large mitochondria in the two liver fractionations reported in Table II was very low, approximately 2 per cent, whereas the small mitochondria contained about 25 per cent of the total tissue PNA. This finding is compatible with the effects of ribonuclease on mitochondria reported by Randavel-Vendrely (11) and by Zollinger (16). The higher PNA content of mitochondria reported by other investigators (9, 13) may be due to less complete separation of large and small mitochondria.

The approximate relative quantities of the particulate fractions within the liver can be estimated by combining data from fractionations in which no material was discarded but in which the small mitochondrial fraction was extensively contaminated with large mitochondria (Table II), with data obtained from purified fractions. On this basis, the approximate quantities of the three particulate fractions in mg of protein-nitrogen per g of fresh liver are: large mitochondria 4.26; small mitochondria 2.80, and microsomes 3.92. Since the cytoplasmic particulates of other tissues have not yet been obtained in pure form, the relative amounts of these cell constituents cannot be calculated for other tissues.

The two mitochondrial sub-fractions prepared from pancreas, sub-maxillary gland, adrenal, and several tumors are probably homologous with those of rat liver, since in these tissues also the mitochondrial pellet occurs as two layers, the particles of the densely packed layer being larger than those of the fluffy layer and containing very much less PNA than the fluffy layer particles. Although elucidation of the relationship of the mitochondrial sub-

fractions in other tissues with those of rat liver will require a thorough study of purified preparations, the findings of the present report indicate that the existence of more than one kind of mitochondria is not limited to rat liver.

SUMMARY

The mitochondrial pellet obtained from rat liver by the fractionation procedure of Hogeboom *et al.* (5) consist of a densely packed lower layer containing mitochondria, overlaid by a loosely packed fluffy layer. By repeated washing and resedimentation the fluffy layer material has been separated from the mitochondria of the dense pellet and from the microsomes. This isolated fluffy layer material consists almost exclusively of particles which are distinctly different from the mitochondria of the dense layer and the microsomes. They are somewhat smaller than the mitochondria of the dense layer, but their similarity to these mitochondria in shape, osmotic behavior, and succinoxidase activity, suggests that they are mitochondrial in nature, and we have therefore called them "small mitochondria". However, in contrast to the large mitochondria, which have an extremely low PNA to protein-nitrogen ratio (less than 10 per cent of that of the microsomes), the small mitochondria have a ratio more than 80 per cent of that of the microsomes. Their alkaline phosphatase activity is approximately twice that of the large mitochondria or microsomes, but their phospholipid content is intermediate.

Large and small mitochondria, apparently similar to those of rat liver, have been obtained from a number of tissues.

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ELECTRON MICROSCOPIC INVESTIGATION OF THE CELL WALL ORGANIZATION OF WOOD

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Numerous photomicrographs of isolated cellulose fibers and tissue sections using ordinary light and polarized light, as well as X-ray investigations, have led to much fundamental knowledge concerning the fine structure of the cell walls of plants. Electron microscopy now makes possible direct observation of the more minute structural elements. Thus, the submicroscopic cellulose fibrils from which the plant cell wall was supposed to be formed were postulated by Frey-Wyssling (8) after studies based on indirect methods and were observed with the electron microscope in plant tissues disintegrated by such mechanical means as the ball mill and ultrasonics (5, 10, 12, 19, 22). The extent to which observations with the electron microscope record the intact organization of these fibrils in the cell wall is determined primarily by the technique used in making the preparations and only secondarily by the resolving power of the electron microscope. Thus, this investigation depends on the preparation of surface replicas or the direct microscopy of sufficiently thin tissue sections.

With the aid of replicas, Preston *et al.* (17) examined the cell-wall structure of *Valonia ventricosa* and Fischbein (6) and Varossieau (23) that of wood tissue. Mühlethaler *et al.* (9, 13, 14) were able with a Waring Blendor to disintegrate different plant fibers into the thinnest cell-wall fragments, leaving an intact fine structure which could be penetrated ideally with electrons. Electron micrographs obtained by both methods have shown clearly that the submicroscopic fibrils represent a real structural element and demonstrate how these fibrils are associated to form the characteristic structural pattern.

The present paper describes an investigation with the electron microscope of sections of wood tissue cut according to the improved microtome techniques developed for electron microscopy of biological materials (15). By employing this method, sections transverse, tangential and radial to

the axes of the cells may be systematically observed, which is necessary in order to clarify the cell wall structure. They go far in demonstrating the original textures of the different wall layers into which the cells are organized without separation of these layers from one another and without detaching the cells from their natural associations. A further comparison with the earlier results obtained by optical polarization and X-ray methods is now possible.

In this paper electron micrographs of sections of the wood of balsa (*Ochroma lagopus*) and of aspen (*Populus tremuloides*) are presented. Many more would be needed to illustrate adequately the complete structural detail of even one type of wood.

METHODS

Cubes of wood measuring about 1 mm per side were placed in cold 18 per cent sodium hydroxide solution (0° C) for a few days for the extraction of the hemicellulose. The samples were then washed carefully and, after dehydration with pyridine, were embedded in polymethacrylic acid ester and cut according to the methods of Newman, Borysko, and Swerdlow (15). The sections were fixed on formvar membranes, and the embedding medium was then extracted with amyl acetate or isoamyl alcohol. Acetone was also used as an extraction agent, but then the plastic membranes were first placed in an electron beam for a short time to render them insoluble in acetone. After the extraction, the sections were shadowed with palladium.

RESULTS

To prevent a maceration of the cells, the lignin component, which is concentrated chiefly in the middle layer, was not extracted.[1] Sections of the wood tissue, free of hemicellulose, were made in the three principal directions; namely, transverse, tangential, and radial. The secondary walls appeared practically free of noncellulose parts, leaving the cellulose network accessible for direct observation. These investigations give little information concerning the cellulose texture in the primary wall, which could hardly be distinguished as an independent structural component in the woody middle layer of the preparations examined here.

[1] In the present classification of the different layers which make up the plant cell walls, the proposals of Kerr and Bailey (11) and of Frey-Wyssling (7) are followed. Accordingly, in this paper the differentiation between the intercellular cement substance or middle lamella, the primary wall and the secondary wall is observed. However, as is also proposed by Frey-Wyssling, the middle lamella and primary wall are grouped together under the title of middle layer.

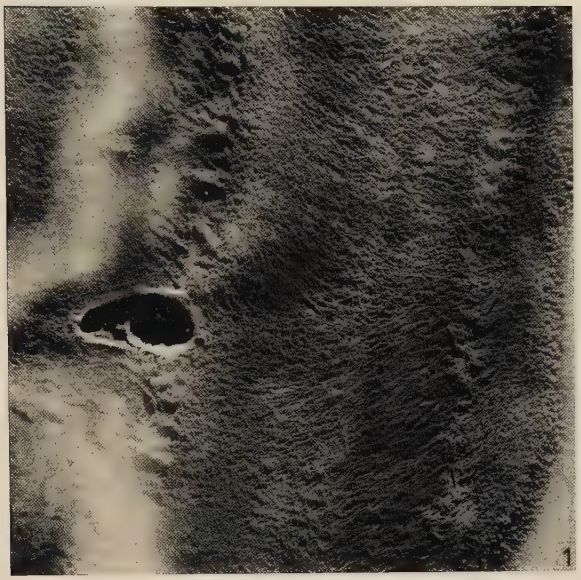


Fig. 1. Balsa. Longitudinal section of fiber. 19 000 ×.

Fig. 2. Balsa. Transverse section of fibers. 30 000 ×.

Fig. 3. Aspen. Longitudinal section of fiber. 6 400 ×.

Fig. 4. Aspen. Transverse section of fibers. 24 000 ×.

In longitudinal sections of the fibers of balsa and aspen, a lamellar stratification of the elementary fibrils in the secondary walls appears (Figs. 1 and 3). In these lamellae the fibrils are generally parallel. The stratified

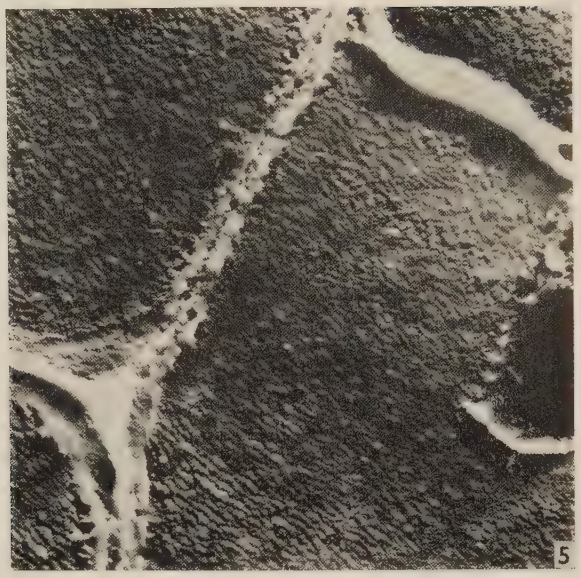


Fig. 5. Aspen. Transverse section of fibers. 8 000 ×.

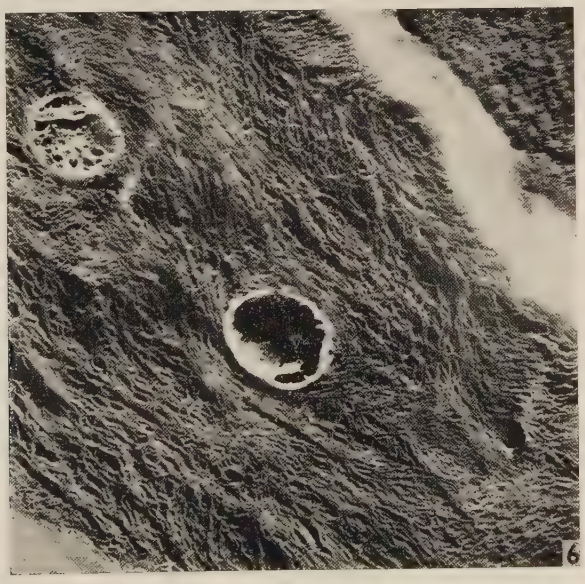


Fig. 6. Aspen. Longitudinal section of medullary ray cell. 13 000 ×.

lamellae may have a different orientation depending on the direction of their fibrils. The illustrations clearly support the opinion obtained indirectly from the optical methods that the secondary walls of the wood fibers can

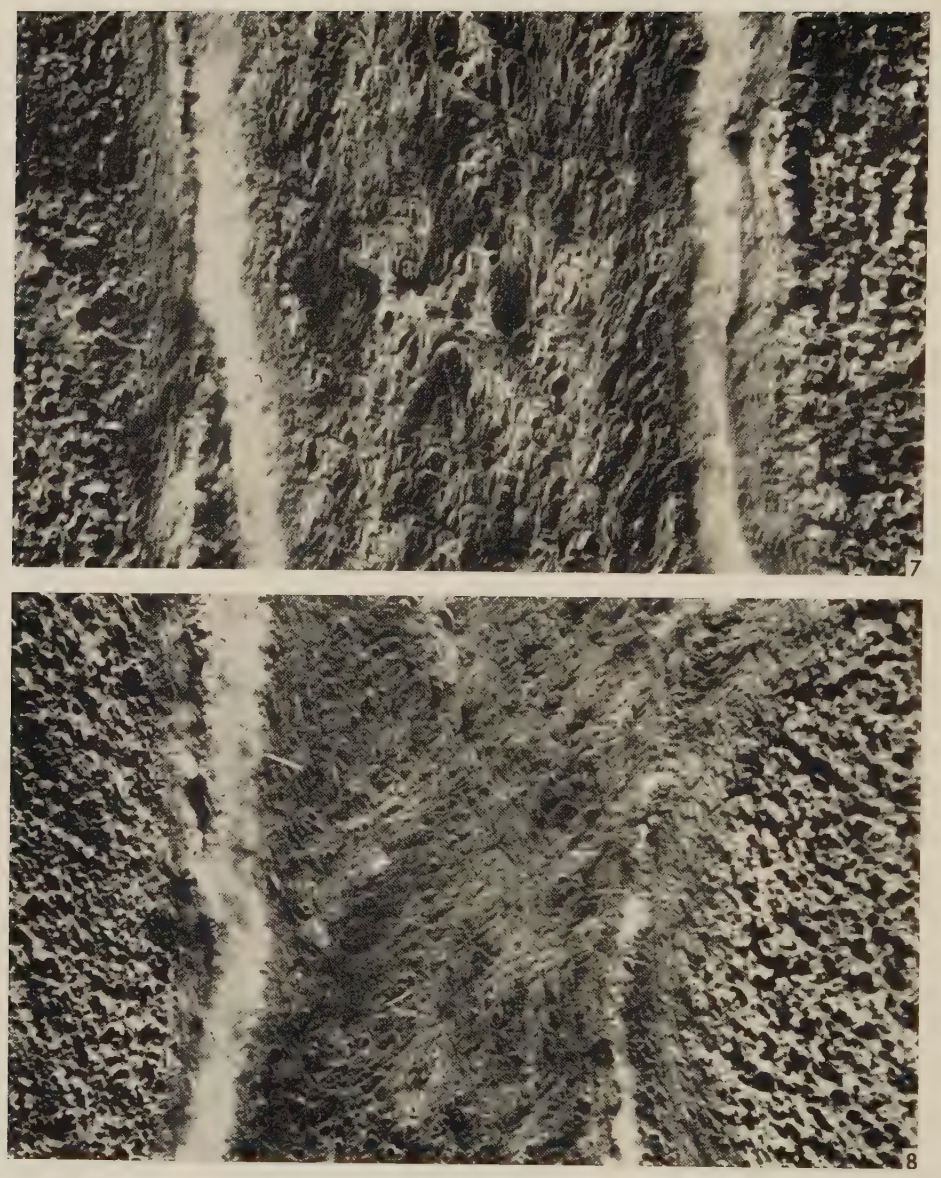


Fig. 7. Aspen. Longitudinal section of medullary ray cell with adjacent wood fibers in transverse section. 22 000 ×.
Fig. 8. Aspen. Longitudinal section of medullary ray with adjacent wood fibers in transverse section. 22 000 ×.

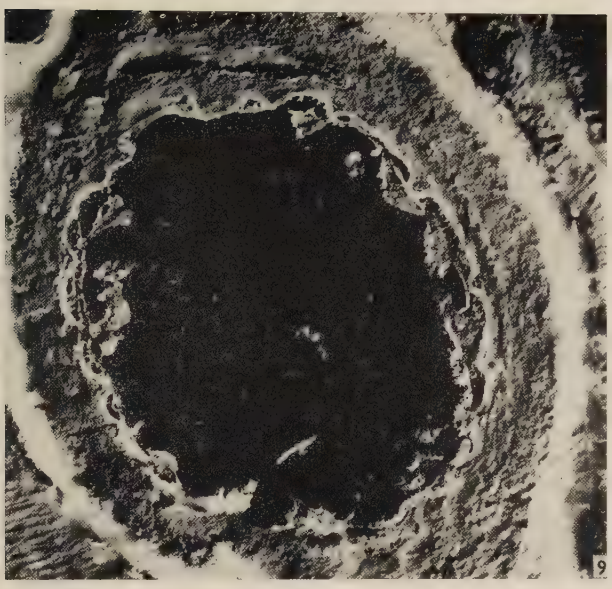


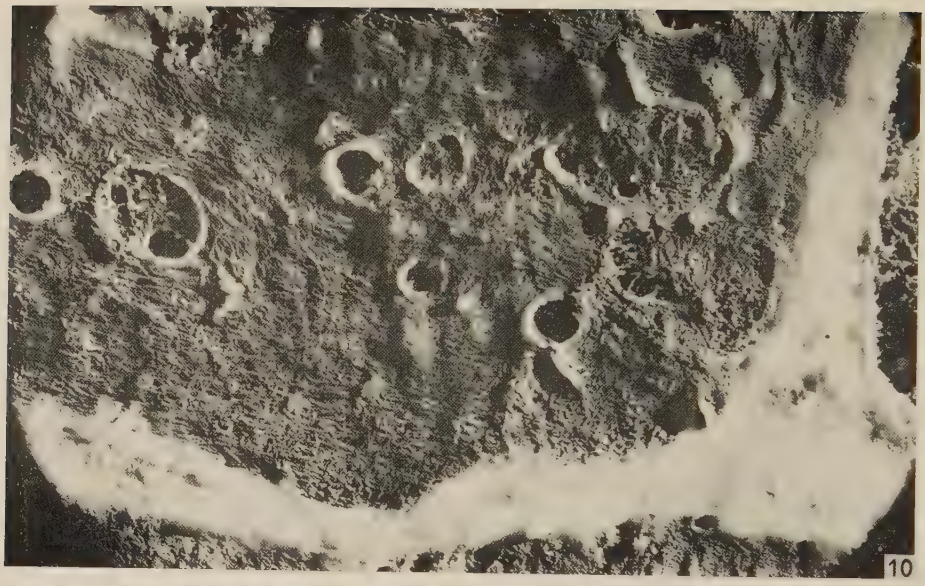
Fig. 9. Aspen. Transverse section of medullary ray cell. 9 000 ×.

be formed of layers in which the crystalline regions of the cellulose assume a twisted, counterrotating course.[1]

Before the illustrations are described in more detail it should be emphasized that it would be incorrect to measure the slope and intersecting angles of the fibrillar layers on the electron micrographs in order to get an accurate measure of the orientation of these layers. The observations made in the present study agree with the optical and X-ray findings of Bailey and his co-workers (1, 2, 3) and of Preston and Wardrop (16, 18, 24) in demonstrating considerable differences in the organization of the secondary wall, not only within the same type of cell but also in different parts of a single cell.

Fig. 1 shows a longitudinal section of the secondary wall of a fiber of balsa. Two differently oriented layers of fibrils can be observed. On the assumption that the middle layers (shown in the figure as less easily penetrated by electrons) run parallel to the axis of the fiber, the two counterrotating layers would have the same slope. The structure shown in a transverse section through the walls of two adjacent fibers of balsa (Fig. 2)

[1] Compare to this the structural principle of conifer wood fibers postulated many years ago by Scrath, Gibbs, and Spier (21).



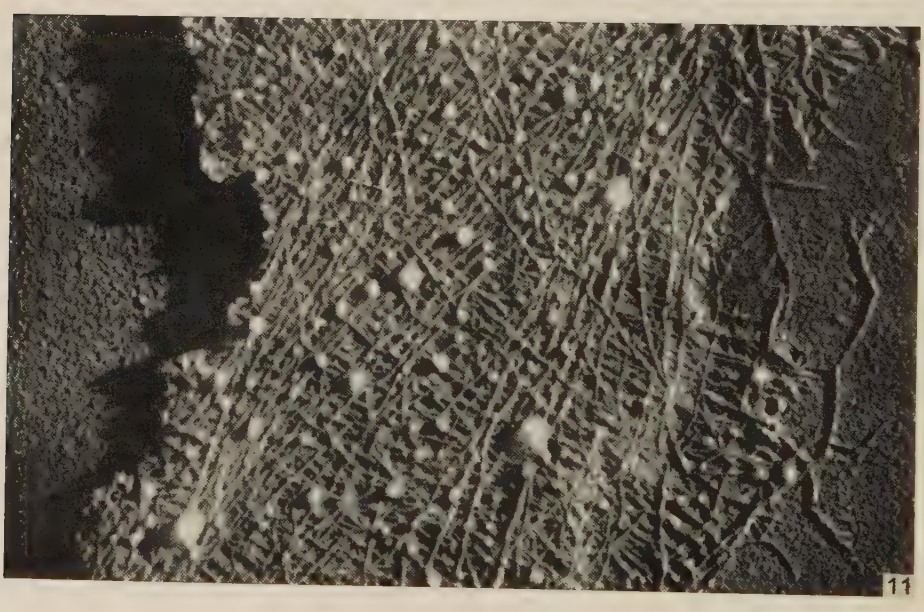


Fig. 10. Aspen. End wall of a medullary ray cell. 14 500 ×.
Fig. 11. Spruce. Longitudinal section of a fragment of fiber. 29 000 ×.

agrees with that shown in longitudinal section. Concentric lamellae indicated by previous optical observations could not be seen, probably because of distortions brought about in the preparation of the section. As will be shown later, such distortions can be especially distinct in transverse sections of fibers.

The micrographs of the longitudinal sections of the fibers of aspen indicate the presence of secondary walls containing layers of fibrils ranging from those oriented at narrow angles to the longitudinal axis to those parallel to the axis. In Fig. 3, for example, in the fiber apex there is a fibril layer inclined at a slight angle from the longitudinal axis; in the lower left corner there is a layer which runs parallel to the fiber boundaries and then bends at a narrow angle away from the fiber axis. The same photograph shows another layer that is approximately perpendicular to the axis of the fiber. Although the slope of the steeply rising fibrils can vary greatly, the possibility is introduced that they belong to an outer layer running perpendicular to the axis of the fiber or as a flat spiral.

The transverse section of the fiber of aspen (Fig. 4) shows a granular structure, i.e., an irregular aggregation of particles with an average size of a few hundred Ångström units. Such a structure is to be expected when a bundle of elementary fibrils is cut perpendicularly or obliquely to their long axes.

In the preparation of transverse sections, the original organization of the fibrils in the secondary wall can be seriously distorted by the action of the microtome knife. When this happens (Fig. 5), the transverse sections seem to be divided into lamellae which, however, always lie perpendicular to the direction of the cut, i.e., parallel to the knife edge. This artefact is obviously restricted to the sectioning of cell walls with compact and more or less steeply ascending fibrillar systems. At the upper right (Fig. 5) a pit canal from the cell lumen runs through the secondary wall to the middle layer. This canal resembles a tube, the transverse section of which is seen as it opens into the lumen.

Occasionally in the transverse sections a characteristic outer zone can be observed delimiting the more opaque middle layer; this was presumed to be a thin layer of fibrils running at right angles to the main axis or slightly twisted about it. This appears most clearly in transverse sections of the fibers which border on the medullary rays (Figs. 7 and 8). This zone is most obvious in Fig. 7 where the medullary ray and wood fibers appear somewhat torn apart, thus giving a most objective identification of the differently oriented outer layer of the secondary wall. These observations agree

with the light-optical findings in coniferous tracheids, indicating that the micellae in the outermost layer of the secondary wall are more flatly twisted than in the deeper principal layer (3, 11, 21, 24). The electron micrographs thus far obtained of the fibers of aspen have given no indication, however, of an innermost layer with flatly winding fibrils, as was described for the conifer tracheids.

Figs. 6, 7, and 8 show transverse sections of the wood of aspen with the medullary cells running radially in a single layer. The secondary walls of these parenchymatous cells sometimes give the impression that they are formed of differently oriented fibril layers. Usually only one layer can be detected which follows the general direction of the long axis of the cell (Figs. 6 and 7). An example of a differently oriented fibril layer appears in Fig. 8. Within the individual layers the course of the fibrils usually does not appear to be closely parallel, and considerable deviations from the general direction can be observed.

In a tangential section of the wood of aspen, a medullary ray cell in transverse section shows transversely and obliquely cut fibrils of the secondary wall (Fig. 9). Fig. 10 is also a tangential section of the wood of aspen and shows an end wall of a medullary ray cell perforated by numerous simple pits which connect the cells radially.

In the longitudinal sections of the medullary ray cells, small pits can also be observed (Fig. 6). These pits, appearing circular to elliptical in transverse section, are on the axial contact-faces of adjacent medullary ray cells. One of the pits in Fig. 6 contains a fibrillar net, as is often observed. Presumably such a net represents the so-called primary membrane of the pits.

In our preparations, the pit openings appear to be bounded by a wall which is not readily penetrated by electrons (Fig. 6, cf. also Fig. 10). This pit wall, which possibly consists of a cellulose network permeated with a highly dispersed noncellulose material, contrasts sharply with the structure of the cell wall surrounding the pits.

It is generally assumed on the basis of investigations with polarized light that the micellae take a circular course around the opening of the pits they enclose. The significance of the circular structure of the pits in relation to the structure of the layers of the cell wall in which the pits occur has come under much discussion.

Even though the electron-microscopic data must be considerably expanded to deal more closely with this problem, the structure of the pits of the medullary rays discussed here are not too closely related to the struc-

ture of the secondary wall. As is shown by Fig. 6, for example, the course of the fibrils of the secondary wall is hardly influenced at all in the immediate vicinity of the pits; this gives the impression that the pit is a structural element completely independent from the fibrils of the secondary wall. In other photographs a displacement of the fibrils of the secondary walls can be observed around the opening of these pits. Viewed in polarized light between crossed Nicol prisms such a displacement of the fibrils of the secondary wall around the pit emphasizes the possibility that the pit could have its own circular arrangement of fibrils. Frequently pits with elliptical transverse section occur in which the long axis of the ellipse is always parallel to the long axis of the cell and hence to the direction of the fibrils of the secondary layer. Even in this case the photographs show hardly any deformation of the pit by the surrounding fibril structure, or vice versa.

In view of our results with the electron microscope, the optically denser wall layer, which delimits the pit opening and the fine structure of which still cannot be interpreted, would be responsible for a circular structure. In addition, fibrils occasionally deflected in the region of the pit opening may superimpose optical effects. Therefore, there is agreement with the idea of Bucher and Wiederkehr (4) who, by light-optical investigations, modified the older ideas of Ritter and Chidester (20) as well as those of Scrath *et al.* (21).

Electron micrographs made of sections of the tracheal walls and longitudinal parenchyma of aspen as well as of sections of wood of fir and spruce must be omitted for lack of space. In all cases the cellulose was observed as submicroscopic fibrils indistinguishable from those already shown. Sections made of loose fibrillar tissue, when photographed, give the clearest indications that the thickness of the fibrils is in the order of 100 Å. Such a photograph is Fig. 11, which shows a fiber fragment, found in a longitudinal section of delignified spruce.

SUMMARY

Electron micrographs were made of thin sections of the cell walls of several kinds of wood. Such sections, cut in different selected directions, permit observations leading to an explanation of the organization of cellulose fibrils in the cell walls. Results are compared with the concepts of submicroscopic micellar structure which have been obtained by means of indirect methods.

These studies were carried out in the laboratory of Dr. Ralph W. G. Wyckoff within the framework of the cellulose investigations in Uppsala which are under the direction of Prof. The Svedberg. The author wishes to express his gratitude to Prof. Svedberg and to Dr. Wyckoff for their encouragement and interest in this work; also to Dr. Wyckoff and Dr. George Rozsa for helpful advice during the investigations with the electron microscope.

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ELECTRON MICROSCOPIC STUDIES OF SMALL CYTOPLASMIC PARTICLES (MICROSOMES)[1]

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Since the work of Bensley and Hoerr (1), isolation of cellular components by differential centrifugation has become a more and more important procedure. This method has contributed much to our knowledge of cell structure and physiology. Although microsomes were conceived of and even named over seventy years ago (7), material evidence for their existence was first obtained in 1940 (6) when Claude isolated them by differential centrifugation. Since that time a rather large amount of work dealing in part with microsomes has been published. The investigations to be described here were undertaken for three main reasons. In the first place it seemed advisable to have some morphological characterization of the material being studied. Secondly, since many investigators use methods which differ in some way from those of others a means for checking the identity of the material was necessary. Finally, it was felt that structure might give some clue to function, and that knowledge of morphology might make possible adaptation of some of the methods of cytochemistry.

Since these experiments were to be followed by studies of enzyme activity they were designed throughout to sacrifice high yield to speed and purity. It might be suspected that time and numerous manipulations would have a considerable effect upon the particles. That such is the case has been borne out by the studies of Klein, Kurnick, and Klein (9), Malmgren *et al.* (11), and Berthet and de Duve (2).

Four criteria were followed in establishing the isolation procedure. 1. Speed—The material must be exposed to an unfavorable environment and to the action of enzymes and other deleterious factors for a minimum amount of time. 2. Purity—The preparations must be as free as possible from recognizable cellular components other than microsomes and anything evi-

[1] These investigations were done during the tenure of fellowships from the Sloan-Kettering Institute and the National Cancer Institute, of the National Institutes of Health, Public Health Service. The work was supported in part by grants from the American Cancer Society; the Lilla Babbitt Hyde Foundation; and the National Cancer Institute, of the National Institutes of Health, Public Health Service.

dently non-biological. 3. Reproducibility—Preparations with similar characteristics must be readily obtainable. 4. Normalcy—Since it is not possible with the electron microscope to determine directly a normal or physiological condition for most biological elements the material was determined to be macroscopically homogeneous and free from amorphous material. Upon fulfilling these qualifications the appearance in 0.88 M sucrose was arbitrarily called "normal."

Most of this work was done with livers from the Heston subline of C3H strain mice. Also C57 black, C57 brown, and A strain mice were used. The results with other strains were indistinguishable from those with C3H mice.

EXPERIMENTAL

The entire isolation was performed at 0–4° C and as rapidly as possible. The animal was killed in the cold and all equipment and fluids were pre-cooled to this temperature. The mouse was killed by severing the spinal column. The liver was immediately exposed and the inferior vena cava cut. Chilled suspending fluid was forced through the liver with a small syringe in order to flush out the blood and chill the tissue as rapidly as possible. The liver was washed in a large beaker of suspending fluid and any obvious pieces of connective tissue (mesenteries, blood vessels, etc.) or blood clots removed. Next the liver was cut in pieces roughly 5 mm square and put into the homogenizer. This was in the beginning a Potter-Elvehjem glass homogenizer. Later, at the suggestion of Dr. Potter, a lucite homogenizer with a stainless steel shaft for driving the pestle was used. The tissue was homogenized with nine volumes of suspending fluid. Originally it was determined that 30 seconds was about the shortest time that would provide a sufficient yield. However, data on the Waring Blendor published by Harman (8) suggest that somewhat longer times would not greatly increase the amount of fragmentation of the mitochondria. Consequently 45 seconds was used in all these experiments. The material so obtained was checked for undue fragmentation by microscopic examination.

The homogenate was then centrifuged. For the lower speeds an International Equipment Company model PR-1 refrigerated machine was used with the multispeed attachment (heads No. 295 or 296). For the higher speeds a Spinco model "L" refrigerated ultracentrifuge was used (rotor No. 40). The centrifugation scheme is discussed below. All tubes are lustron except those for IEC No. 295 which are glass.

Resuspension of the sticky gelatinous pellet following high centrifugal forces presented a problem. This was at first done as follows: the pellet was covered with 1–2 ml of suspending fluid; the pellet was carefully pulled away from the sides of the tube then drawn in and blown out of a fine bore pipette until no large fragments could be seen. There are obvious objections to this method. In the majority of experiments resuspension was done with a lucite pestle with a stainless steel shaft. The pestle fit the centrifuge tubes loosely (1 mm tolerance). The particles were quickly and easily resuspended. No morphological differences could be seen following the two methods.

FIG. 1. CENTRIFUGATION SCHEME

Treatment	Duration of treatment (minutes)		
	0.88 M sucrose	0.25 M sucrose	Chambers' solution
Homogenate			
500 × g	10	6	5
Sed. / Sup.			
10 000 × g	10	6	5
23 000 × g	5	3	3
Sed. / Sup.			
137 000 × g	30	15	14
Sed. / Sup.			
resuspended			
137 000 × g	30	15	14
Sed. / Sup.			
"microsome pellet".			

Sed. = sediment.
Sup. = supernatant.

Three fluid media were used, 0.88 M sucrose, 0.25 M sucrose, and Chambers' solution. These were sometimes buffered with 0.01 M phosphate at pH 7.45. (The cations were reversed from the ordinary Sørensen buffer, *i.e.*, K_2HPO_4 and NaH_2PO_4 were used instead of KH_2PO_4 and Na_2HPO_4. This more nearly approximates the high $[K^+]$ of the intracellular environment.) The components of Chambers' solution are as follows: KCl, 5.2 gm; NaCl, 1.75 gm; sodium citrate, 2.5 gm; distilled water to 1 000 ml (3). By using three different fluids and comparing the results it was possible to estimate the effect of the fluids and the amounts and kinds of artefacts produced by them, if any.

The scheme in Fig. 1 was arrived at empirically by using ordinary light, phase, and electron microscopes to examine the morphological condition of the fraction. It is obvious that the yield is reduced by this technique.

The procedure was first devised for 0.88 M sucrose. The final speed was calculated from a formula derived by Pickels (12) to sediment particles 50 mμ in diameter. The same formula made it possible to vary the duration of centrifugation so that isolation with the other media was as nearly identical as possible. There were too many uncertainties in its use to assume that the formula gave exact information.

The microsome pellet was resuspended to a certain volume and an aliquot was mixed thoroughly with an equal volume of 1 or 2 per cent OsO_4 which was cold, at room temperature, or warmed to 37° C. (Acetic-alcohol and phosphotungstic acid were tried as fixatives. The former left the particles with a very low density and somewhat distorted; the latter was very destructive. Unfixed material gave the same picture as osmium but electron densities were much lower.) After a certain time a drop was put on each of several grids which had been covered previously with a thin film of collodion. They were then washed sufficiently to remove most of the suspending fluid.

Complete removal of the sucrose was assured, since it can be readily recognized under the electron microscope. Finally, the specimen was dried and in some cases shadowed with chromium. Examination and micrographs were made on an RCA model EMU electron microscope. Measurements were made with a calibrated hand lens on prints enlarged from the negatives of a known magnification.

RESULTS

The accompanying photographs illustrate the findings.

Aside from an infrequent mitochondrial fragment, there are three types of particles in the microsome fraction. (Here called large, intermediate, and small particles.) One kind of particle is relatively large (Fig. 2). In 0.88 M sucrose it has an extreme size range of 40–330 mμ with a mode at 125–130 mμ and a mean of 129 mμ. The smallest particle is somewhat denser to the electron beam; the density increases with osmium. It has an extreme size range in 0.88 M sucrose of 8–35 mμ with a mode at 23–25.5 mμ and a mean of 22.2 mμ (Fig. 4). It will be seen from the graphs that its size is very constant. The intermediate size particle has, with osmium fixation, high electron density. It is considerably less dense without osmium. The size range of the particle in 0.88 M sucrose is 30–220 mμ with a sharp mode at 65–70 mμ and a mean of 78.7 mμ (Fig. 3).

All controls have been negative for these three particles. Controls were suspending medium, medium plus OsO_4, OsO_4 solution, medium plus phosphate, medium plus phosphate plus OsO_4, the collodion film, chromium

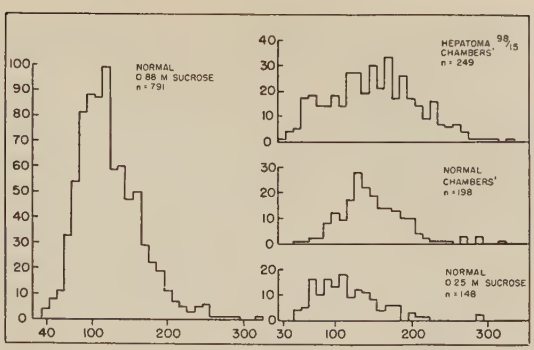
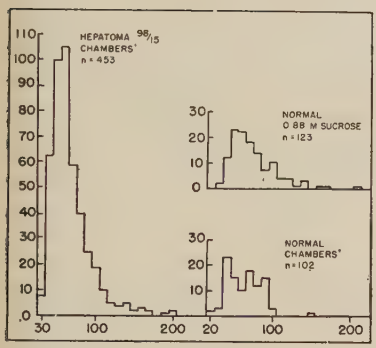


Fig. 2.



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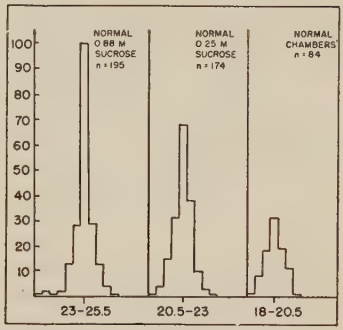


Fig. 4.

Fig. 2. Size distribution of large microsomes. Classes are 10 mμ.
Fig. 3. Size distribution of dense intermediate size microsomes. Classes are 10 mμ.
Fig. 4. Size distribution of small microsomes. Classes are 2.5 mμ.

shadowed films, distilled water, tap water, and also such general controls as cigarette ash and dust from the laboratory in which all preparations were examined.

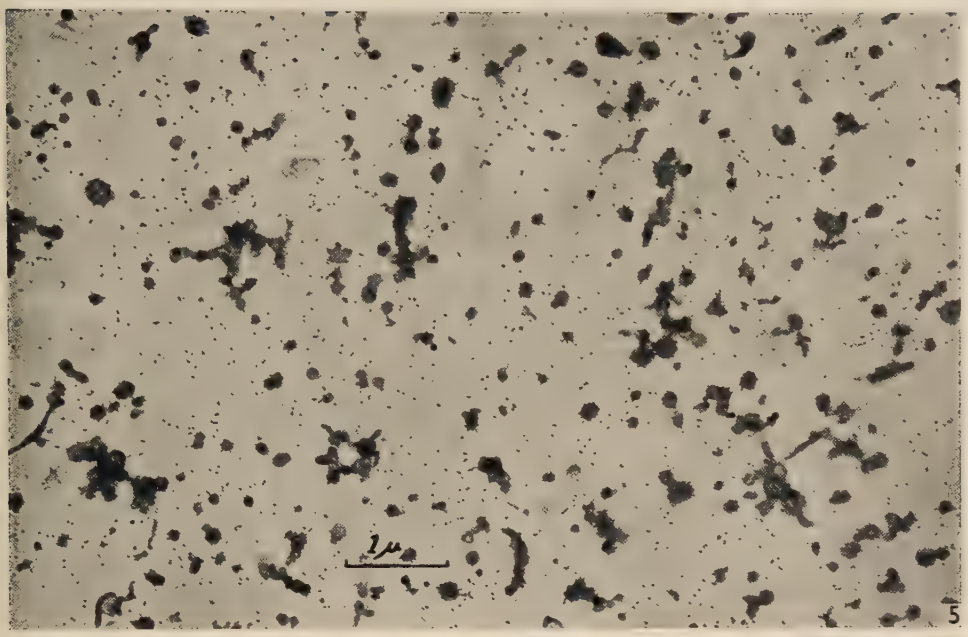
It is very difficult if not impossible to say how nearly the particles of the microsome fraction as prepared here resemble their normal condition in the living cell. Zollinger (14) observed living normal and malignant rabbit and frog cells in hypotonic solution with a phase microscope. Small particles were seen to arise in the cytoplasm evidently swelling from submicroscopic size due to the effect of the environment. They remained just above the limit of resolution of the microscope. These were presumably particles of the microsome fraction. Particles very closely resembling those shown here have often been seen and described in electron micrographs of whole cells

and thin sections of cells. So although direct comparison with the living cell is not possible, the components of the microsome fraction can be found in electron micrographs of intact cells prepared under various conditions. They appear to be identical.

Fig. 5 shows a relatively low power field of the microsome fraction prepared in 0.88 M sucrose and fixed in cold 1 per cent OsO_4. The smallest particle is scarcely visible. Note the tendency to form chains and the apparently sticky nature of the large particles. Fig. 6 is a typical picture of particles prepared in the same way but shown at a higher magnification. About 15 large particles seem to have aggregated into a branched chain. Four or five intermediate size dense particles are present. The smallest particles are especially well shown. They often appear in groups of two or three and may be held together by a sticky substance. It is interesting to note that invariably many appear to be stuck on the surface or held within the largest particles. Frequently the large particles look like highly crenated red cells, so numerous are the small particles on or just beneath their surfaces. These small particles were totally unexpected. Their size, 25 mμ, is only half that which centrifugation was calculated to sediment. They are definitely concentrated in the sediment, since inspection of the supernatant shows there are relatively no more of them there than of the large particles. (Some particles are always present in the supernatant because of convection currents, mixing resulting from accidental shaking etc., and loss from the sediment during decanting.) It would appear that the small particles are carried down by the large ones. They are present in all three media. The size varies slightly but is significantly larger (6 mμ) in 0.88 M sucrose than in the isotonic solutions (Fig. 4). This is contrary to the expected osmotic effect. Instances such as shown in Figure 9 (see arrow), where a large particle seems to have burst open, especially suggest that the smallest particles are normally within the largest. Also suggestive is the fact that when the large particles are stretched out into a tube the small particles are in the center of the tube, and if there are several of them in a chain the axis of the chain is always parallel to the axis of the tube. Furthermore, if the large particles appear to have a concave surface the small particles are around the thick outer edge rather than in the shallow depression.

Upon incubation in the Warburg apparatus for 2 hours at 37° C under oxygen the smallest particles are no longer present (Figs. 11, 12). The smallest particles are present in mouse hepatoma 98/15 and apparently unaltered from their condition in normal liver.

The dense intermediate size particles are also present with all three suspend-



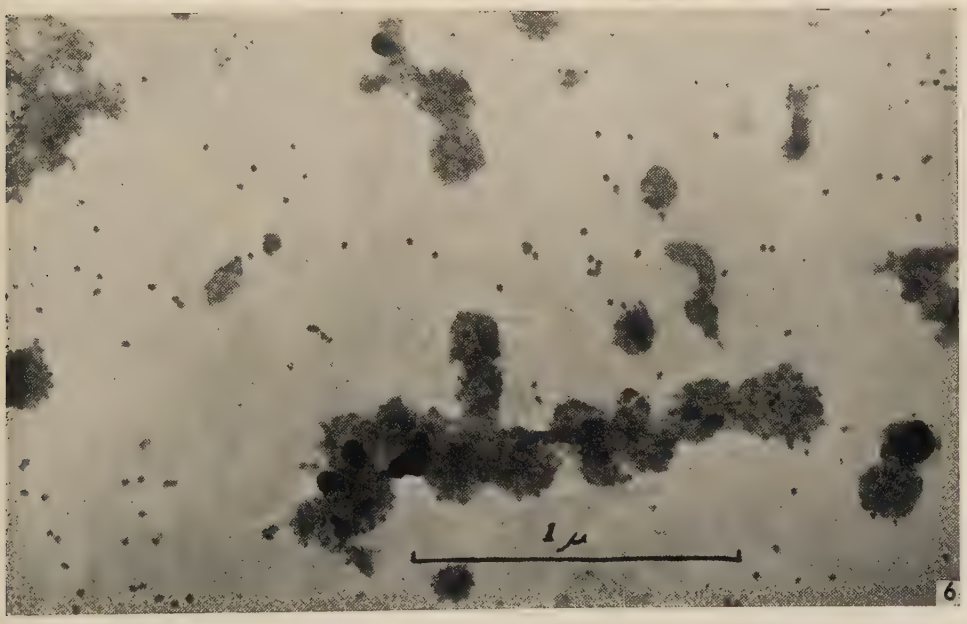
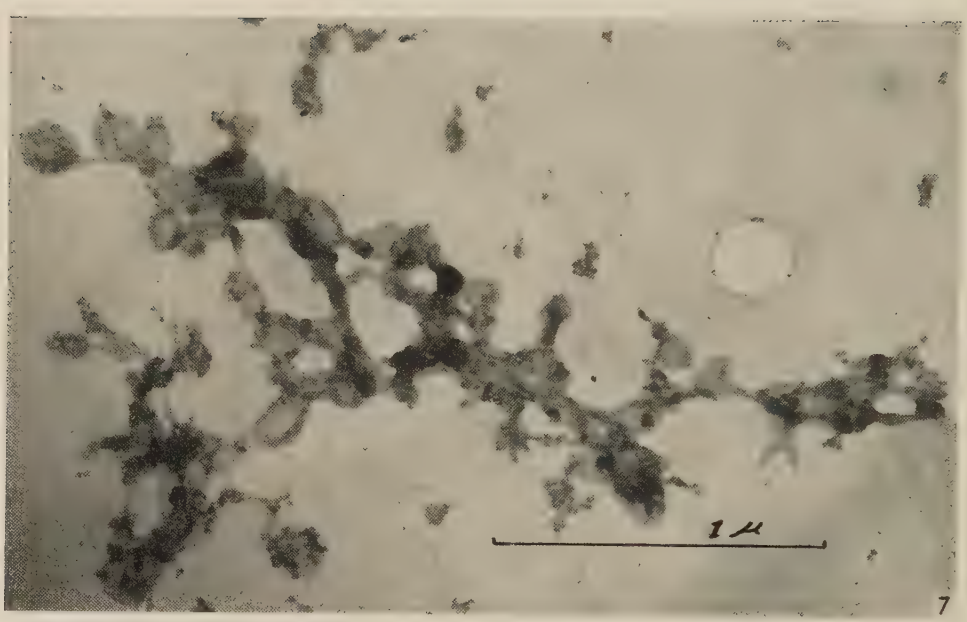


Fig. 5. Microsome fraction prepared in 0.88 M sucrose. Fixed: cold 1 per cent OsO_4. Magnification 14 400 ×.
Fig. 6. Same as Fig. 5. Magnification 45 200 ×.



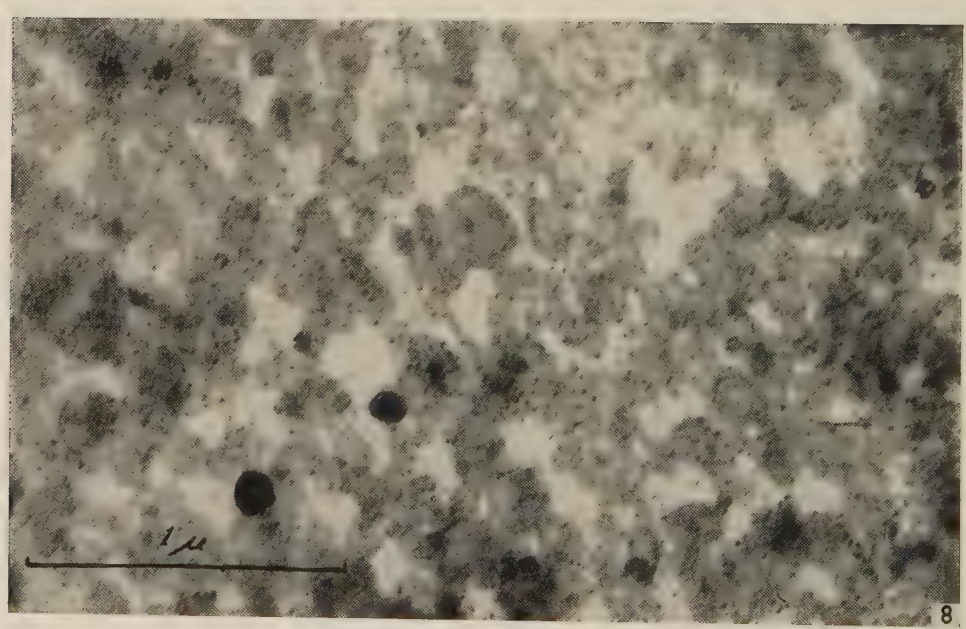
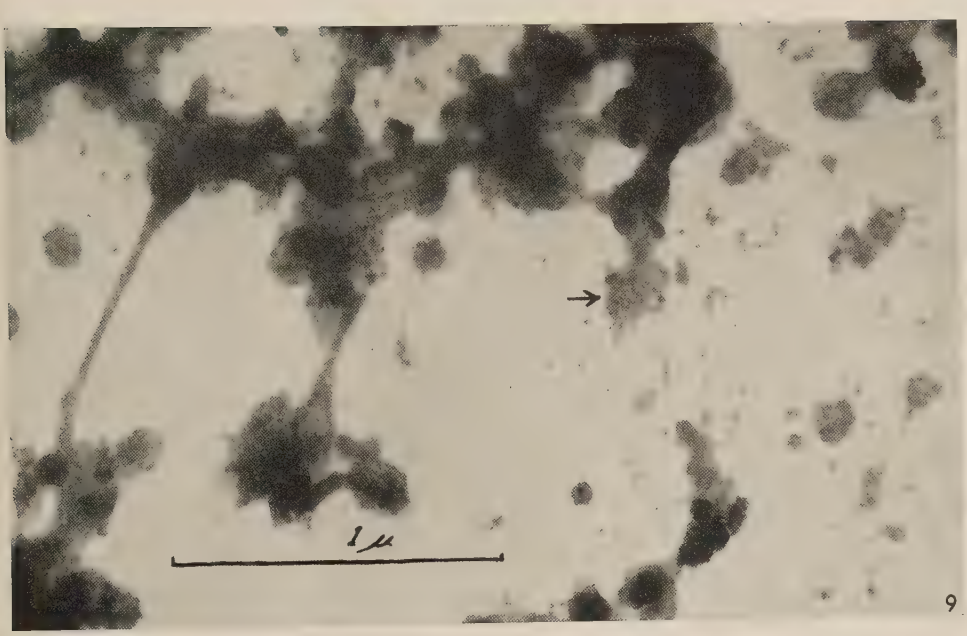


Fig. 7. Microsome fraction prepared in 0.25 M sucrose. Fixed: cold 1 per cent OsO_4. Magnification 45 200 ×.
Fig. 8. Microsome fraction prepared in Chambers' solution. Fixed: cold 2 per cent OsO_4. Magnification 45 200 ×.



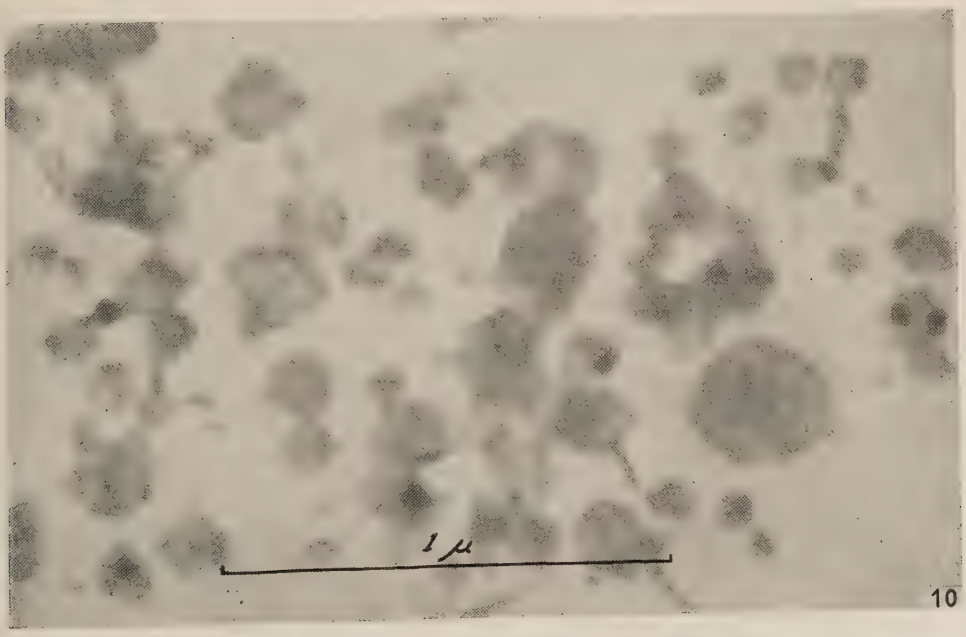
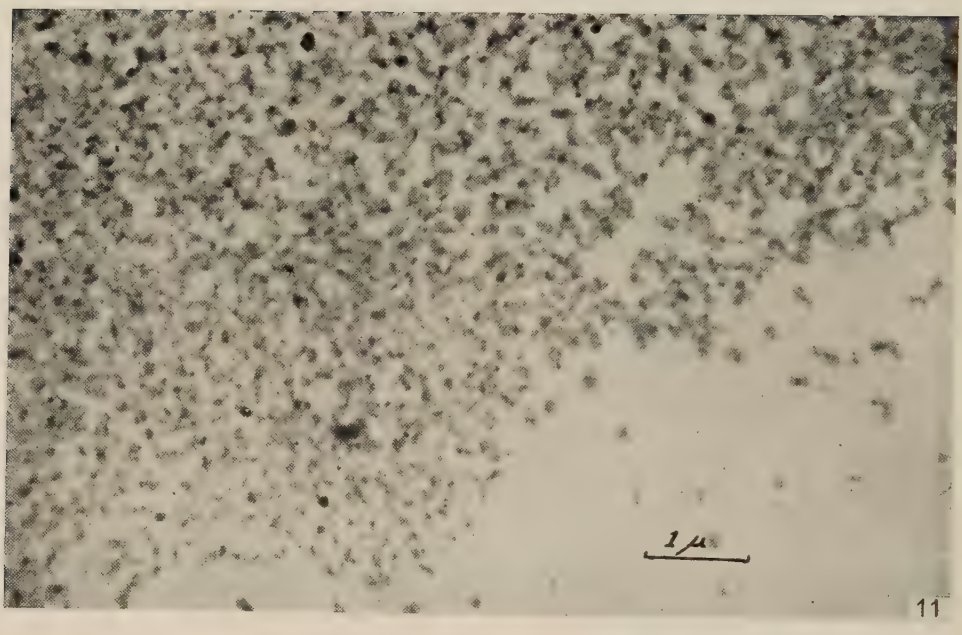


Fig. 9. Microsome fraction prepared in 0.88 M sucrose. Fixed: cold 2 per cent OsO_4. Magnification 45 200 ×.
Fig. 10. Microsome fraction prepared in 0.25 M sucrose. Fixed: 2 per cent OsO_4 at 38° C. Magnification 61 600 ×.



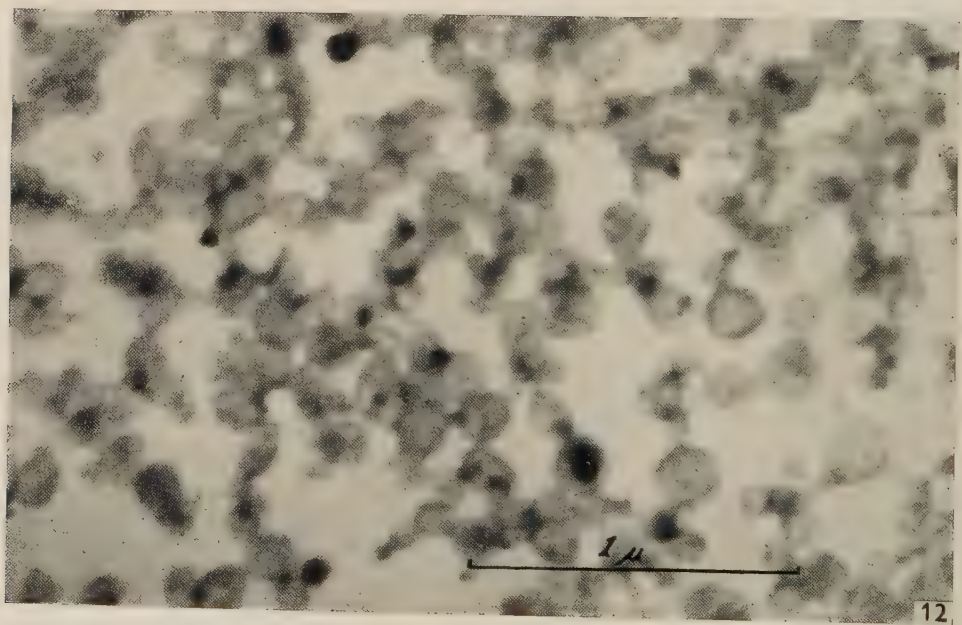
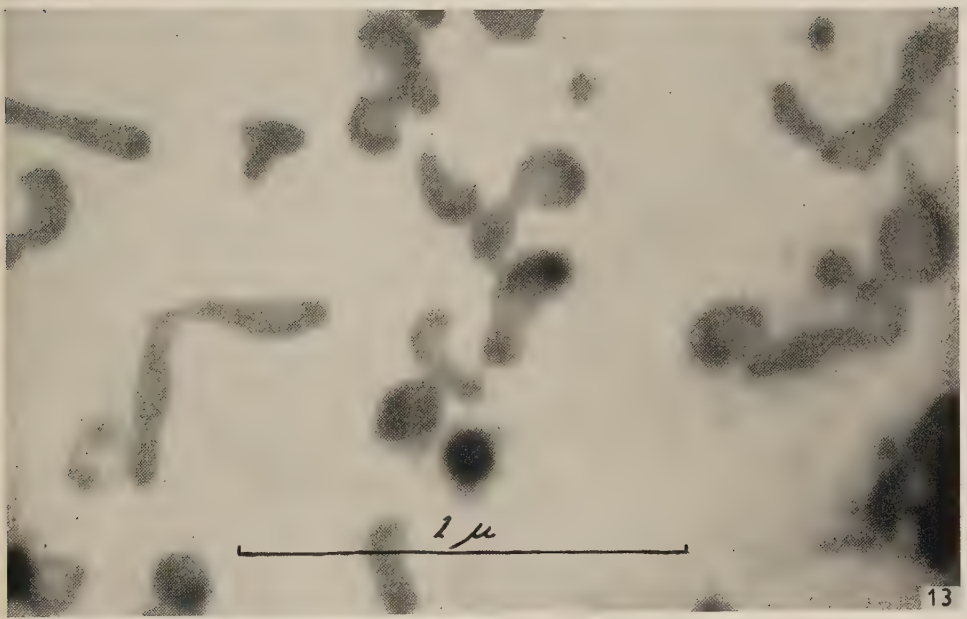


Fig. 11. Microsome fraction prepared in Chambers' solution. Incubated 2 hours in Warburg apparatus under oxygen at 38° C. Fixed: cold 2 per cent OsO_4. Magnification 14 400 ×.
Fig. 12. Same as Fig. 11. Magnification 45 200 ×.



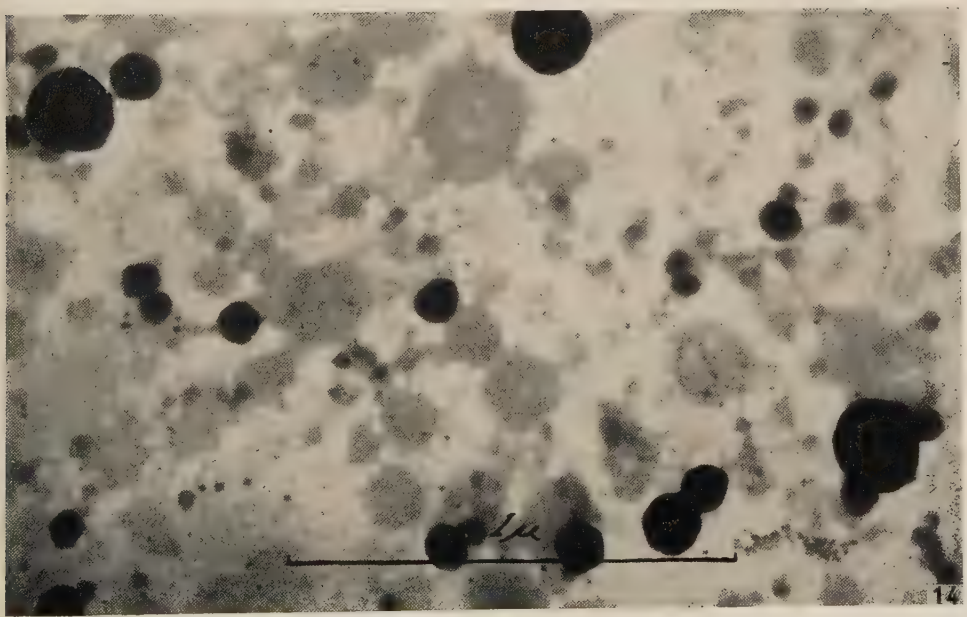


Fig. 13. Microsome fraction prepared in 0.88 M sucrose. Incubated 2 hours in Warburg apparatus under nitrogen at 38° C. Fixed: cold 2 per cent OsO_4. Magnification 61 600 ×.
Fig. 14. Microsome fraction from hepatoma 98/15 prepared in Chambers' solution. Fixed: cold 2 per cent OsO_4. Magnification 61 600 ×.

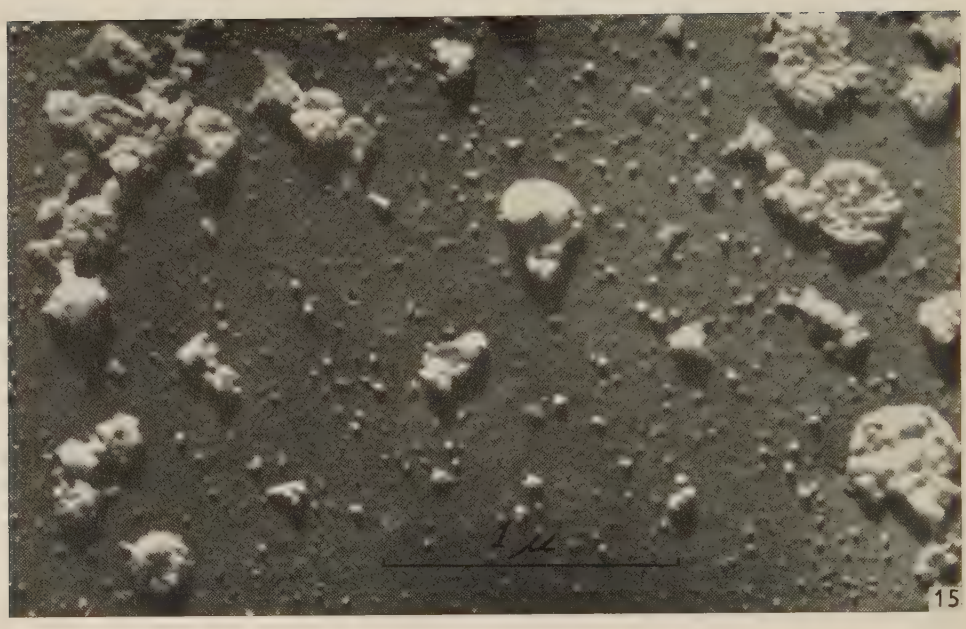


Fig. 15. Microsome fraction prepared in 0.88 M sucrose. Fixed: cold 2 per cent OsO_4. Shadowed with chromium. Magnification 40 800 ×.

ing media but less prominent in sucrose. It is suspected that they have a high lipid content because of their affinity for osmium.

The intermediate size particles are not lost during incubation (Figs. 11, 12).

They appear in appreciably greater numbers in the microsome fraction of mouse hepatoma 98/15 (Fig. 14). The significance of this finding is not understood.

The greatest part of the microsome fraction is occupied by the large particles. Although present with all media they are structurally the most sensitive components. Fig. 6 14 show the range of variation in these experiments. In 0.88 M sucrose the large particles appear to be made of a homogeneous sticky substance, probably protein, of low electron density and low affinity for osmium. Stuck in or on their substance are the small particles. There is little or no suggestion of a limiting surface layer. Quite infrequently in 0.88 M sucrose the surface of the particles appears sharper and suggestive of a membrane. They are sometimes stretched out into tubes or cylinders. The stretching process can be observed during deformation of the collodion film under intense bombardment by the electron beam (although stretching

and bursting of the particles occur without such intervention). These effects are also suggestive of a membrane, as pointed out above.

In Chambers' solution (Fig. 8) the large particles are less dense. Their boundaries are more sharply defined although there is little indication of a membrane unless stretched with the electron beam. Their tendency to form chains is perhaps even more pronounced.

Structurally, 0.25 M sucrose (Fig. 7) has a very deleterious effect on the large particles. Their individual shapes are frequently difficult to determine. Their density is low. They appear to form indistinct strands rather than chains. There are many particles suggesting collapsed membranes. However, the three types of particles can still be demonstrated.

Certainly it cannot be said that there is proof that the large particles possess a membrane but additional strong support is given to this possibility by pictures of suspensions which have been incubated in the Warburg apparatus under oxygen at 37° C. These pictures present a definite collapsed balloon-like form. Note also in Figure 13 the appearance of long tubular forms after anaerobic incubation.

Preparations shadowed with chromium gave no conclusive evidence of a membrane although some pictures were suggestive (Fig. 15). They do show, however, that the small particles are spheres while the large ones are flattened like thick pancakes (undoubtedly a drying artefact). Small particles are seen protruding from the surface but whether inside or out cannot be ascertained. The intermediate size particles cannot be positively identified from shadowed material.

No difference has been found between the large particles from liver and hepatoma except in the size distribution curves. The hepatoma curve is much broader and lower and the poorly defined mode is 40–50 mμ higher (Fig. 2).

Although the chemical composition of the whole fraction has been studied (13) the composition of the individual particles is unknown. It is probable, however, from the work of Chantrenne (4) that the smallest particles contain RNA. The intermediate dense particles probably contain lipoprotein (5). Which are the glycogen particles reported by Lazarow (10) is not known but probably they are larger than these three and are poured off in a buffy layer that sediments above these.

SUMMARY

Submicroscopic particles from the cytoplasmic fraction of mouse liver homogenates have been isolated by differential centrifugation. Electron

microscopic studies show that there are three distinctly different elements. They can be identified by size and by their density to the electron beam. They cannot ordinarily be confused with mitochondria or mitochondrial fragments. The large particles are composed of a sticky substance and probably possess a membrane. The large components probably contain the small ones which are spilled out, evidently unaltered, upon rupture of the membrane. The intermediate particles are homogeneous and lipoidal or at least contain many unsaturated bonds. Chaining is frequent in both the large and the small particles.

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SECRETION OF SPERM AGGLUTININ IN THE FERTILIZED EGGS OF THE SEA URCHINS

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In 1950 the writer (7) showed that the fertilizin, sperm agglutinin, can be detected in the perivitelline fluid of the fertilized eggs of *Strongylocentrotus pulcherrimus,* and that it is the product of the egg. The report given below confirms and extends the previous results of the writer.

METHOD

The eggs of *Strongylocentrotus pulcherrimus* and *Temnopleurus hardwickii* were fertilized in sea water. The eggs were centrifuged two minutes after mixing a drop of sperm suspension with 0.5 ml eggs in 15 ml sea water, when the fertilization membrane was fully inflated. Thereafter they were rinsed with 15 ml 1-*M* urea solution of pH 8 for five minutes. The solution in which the membrane of the eggs was completely removed, was changed three times. The eggs were then allowed to stand in 15 ml sea water. The agglutinin titer of the solution was measured by the drop method. The fresh sperm of *Temnopleurus* was kept in acidic sea water of pH 6 until use, as its capacity of agglutination in sea water of pH 8 is rapidly lost.

RESULTS

The jelly of the unfertilized eggs of *Strongylocentrotus pulcherrimus* was removed with HCl-sea water of pH 5 which was changed four times. The eggs were then fertilized and rinsed with one *M* urea solution to remove the fertilization membrane as described above, and then kept over night in sea water. The control eggs were in the blastula stage. In the series of experiments the concentration of the sperm agglutinin in egg water of the unfertilized eggs and of the first solution of the jelly dissolved in HCl-sea water were 2 000 and 4 000 units respectively. In the supernatant of the fourth washing with acidic sea water the reaction of agglutinin was negative. This shows that the agglutinin in the jelly is removed nearly completely by such a procedure. The urea solution, with which the fertilized eggs were rinsed, showed a slightly positive reaction. On the other hand, the supernatant sea water in which the fertilized and denuded eggs were allowed to stand for

12 hours, showed the positive agglutinin reaction of 128 units. These results reveal that in the eggs of *St. pulcherrimus* the sperm agglutinin emerges even after fertilization.

In the eggs of *Temnopleurus hardwickii* the egg water as well as the jelly solution usually exhibits none or only a slightly positive agglutinin reaction. The urea solutions, with which the fertilized eggs were rinsed, also showed a similar reaction. In contrast to this, the supernatant sea water in which the fertilized eggs denuded of the fertilization membrane were allowed to stand for 12 hours, showed the reaction of 32 units. This reaction was more than 30 times greater than that given by the egg water of the unfertilized eggs. These results, therefore, agree with that in *St. pulcherrimus*, insofar as the fertilized eggs secrete the sperm agglutinin, and the jelly of the eggs of *Temnopleurus* has no close relation with the sperm agglutinin.

In order to study the mode of secretion of the agglutinin in the fertilized eggs of *Temnopleurus*, the following experiment was carried out. Forty-five minutes after fertilization the jelly was removed with acidic sea water of pH 5, and the eggs were then allowed to stand in sea water. All of the eggs hatched after 12 hours and were in the swimming blastula stage. The jelly solution described above was slightly positive in the agglutinin reaction. In contrast to this, the supernatant of the swimming blastulae, which is assumed to contain the perivitelline fluid, was positive in agglutinin reaction, measuring 8 units. The swimming larvae were thereafter cultured in fresh sea water for another 12 hours, where they became young gastrulae. The supernatant of the last mentioned culture was also positive in agglutinin, measuring 32 units. From these results it is evident that the fertilized eggs of *Temnopleurus* actively secrete the sperm agglutinin, not only immediately after fertilization, but also in the blastula and gastrula stages.

DISCUSSION

The fertilizin theory of Lillie (3) aimed at accounting for the specific union of gametes and the resulting activation of the ovum. A "sperm receptor" was assumed to combine with the fertilizin and thus to release the activating effect of the fertilizin within the egg. The fertilizin was found in the supernatant of the egg suspension. According to Lillie's opinion (2, 3, 4) it was secreted from the unfertilized eggs. Loeb (5) suggested that the agglutinin merely corresponds to the dissolved jelly that surrounds the eggs. Most recent workers, Tyler (9, 10) and Tyler and Fox (11) agree that the sperm agglutinin is derived only from the jelly.

In 1950 the writer showed that the unfertilized as well as the fertilized eggs of *St. pulcherrimus* are able to secrete the fertilizin. Byers (1) repeated two of the writer's experiments using *Arbacia* eggs. He showed that the supernatant of the jellyless eggs failed to agglutinate the sperm, and that the supernatant from the fertilized shaken eggs contained sperm agglutinin, but the titer was not higher than the supernatants from the unfertilized control suspensions. In the writer's present experiment, the fertilization membrane was removed in nearly all of the eggs by using the isotonic urea solution. By this procedure the jelly as well as the fertilization membrane could be removed completely. The result was that sufficient quantities of sperm agglutinin for the evidence of secretion were detected in the supernatants of the fertilized and denuded eggs in the two species of sea urchins used. The discrepancy between Byers' and the writer's results may be due to the incomplete removal of the fertilization membrane, and to the fact that the secreted agglutinin will be neutralized by the antifertilizin, which is released into sea water by the eggs broken up upon vigorous shaking. Runnström (8) and Monroy and Runnström (6) actually found antifertilizin in the egg. According to the writer's observation, the secretion of the sperm agglutinin is more distinct in the fertilized than in the unfertilized eggs of *Temnopleurus.* This shows that the egg produces an agglutinin which is distinguished from the jelly coat.

Lillie's explanation on the non-fertilizable condition of already fertilized eggs is that the free fertilizin is bound in some way shortly after fertilization, and that fertilized eggs can then produce no more fertilizin. In view of the writer's results that fertilized eggs secrete agglutinin even in the swimming blastulae, it appears unlikely that free fertilizin is bound after fertilization.

There appear to be two possibilities with respect to the secretion of the sperm agglutinin by the fertilized eggs. One is the gradual release of the cytofertilizin already present in the unfertilized eggs, and the other is the production of sperm agglutinin as a metabolite of the larvae. These two must be studied in relation to the agglutinin and the egg architecture.

SUMMARY

1. Secretion of the sperm agglutinin in the fertilized eggs of *Strongylocentrotus pulcherrimus* and *Temnopleurus hardwickii* was demonstrated in the fertilized eggs in which the fertilization membrane was previously removed with isotonic urea solution.

2. In the normal fertilized egg of *Temnopleurus hardwickii* the sperm ag-

glutinin was detected in the supernatants after hatching of the swimming blastulae.

3. Free swimming larvae of blastula and young gastrula stages of *Temnopleurus* also secrete the sperm agglutinin.

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ROTATING NUCLEI IN TISSUE CULTURES OF ADULT HUMAN NASAL MUCOSA[1, 2]

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Epithelium from the adult human nasal mucosa has been found to emigrate from explants of biopsies (obtained without the use of anaesthetics) in simple hanging drop cultures (6). This method has been used to obtain additional information regarding the behavior of human nasal mucosal cells *in vitro* with a view to exploring allergic phenomena, cf. (5). Using time-lapse cinematographic technique with phase contrast microscopy, cf. (3, 4), more than 1 000 feet of 16 mm film records have been prepared. While viewing films for a description of the activity of wandering cells, cytoplasmic movements at the margin of the mucosal sheet especially in relation to pinocytosis (cell drinking), and other phenomena, it was frequently noted that the nuclei of the epithelial cells displayed rotational movements. These were generally in the same plane as that of the cover slip and occurred in both clockwise and counterclockwise directions. Since nuclear rotation was seen in elements with typical mobile filamentous mitochondria, the phenomenon was not believed to represent an aspect of degenerative change. This present paper is designed to report these observations in some detail.

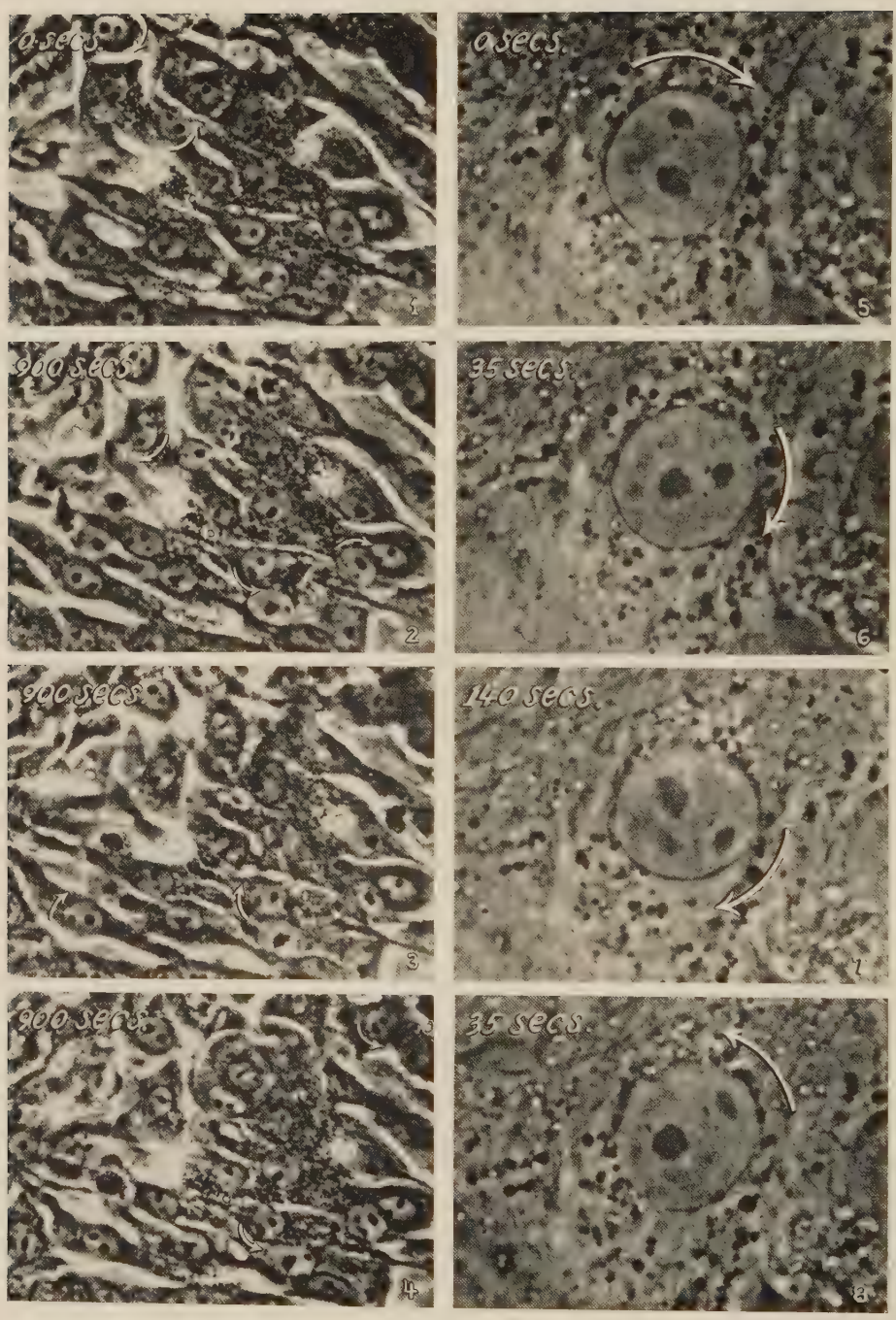
MATERIALS AND METHODS

The culture technique employed was the same as that described by Rose, Pomerat and Danes (6). Motion picture records were made both at 1 and 8 frames per minute with dark phase contrast 8 and 1.9 mm objectives (AO). When these were projected at the standard rate of 16 frames per minute, the acceleration of activity was 960 and 120 times, respectively.

The motion picture films were analyzed with the use of a Leitz photo enlarger. Nuclei of active cells were projected from individual frames onto tracing paper, utilizing the cine film perforations as reference points for alignment. The nuclear

[1] Aided by a contract from the Department of the Navy (N6onr-266) and a grant from the Southwest Allergy Forum.

[2] With the technical assistance of C. George Lefeber and McDonald Smith.



Figs. 1–8. Enlargements from film frames of living adult human nasal mucosa in tissue culture. Phase contrast microscopy. The time lapse between successive frames is given in seconds at the upper left of each photograph. Arrows indicate the direction of movement.
Figs. 1–4 show clockwise and counterclockwise nuclear rotation in a field of outwandering epithelial cells. Magnification approximately 350 ×.
Figs. 5–8. The progress of an individual nuclear rotation in both a clockwise and a reverse direction. Taken from a portion of the film used in the construction of Fig. 9. Magnification approximately 1500 ×.

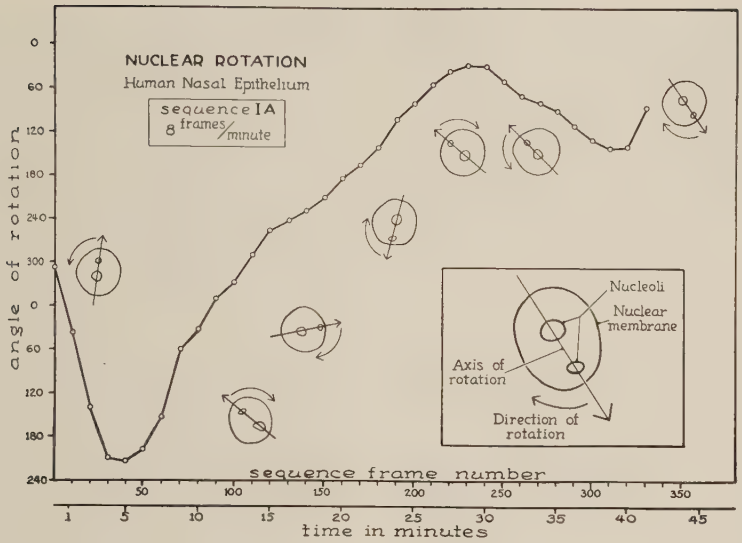


Fig. 9. Method of analyzing cinematographic records of cells from the human nasal mucosa in tissue culture. Using a Leitz photo enlarger, tracings were made of nuclei and nucleoli of active cells. With the indications given in the inset, the direction of rotation was determined by means of a protractor to produce the graph. This shows two clockwise and two counterclockwise movements of the same nucleus within a period of 45 minutes.

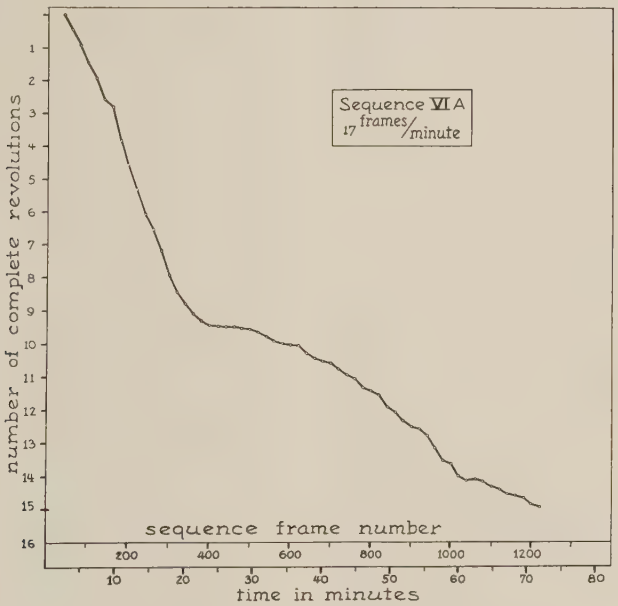
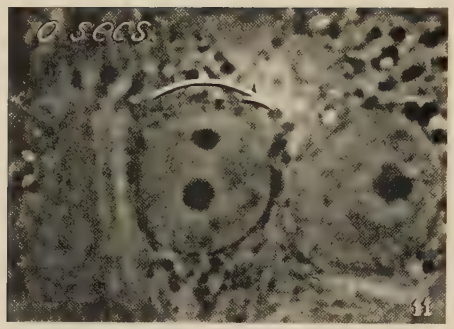


Fig. 10. Graph prepared with the technique described in Figure 9 summarizing the film sequence containing the nucleus which showed the most frequent and the most rapid rotatory activity. All movements were in a clockwise direction. Filamentous mitochondria could be seen in this cell while its neighbor gave no evidence of nuclear rotation.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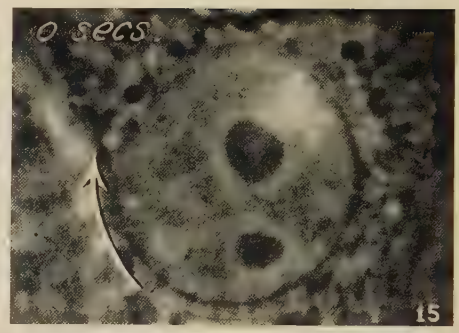
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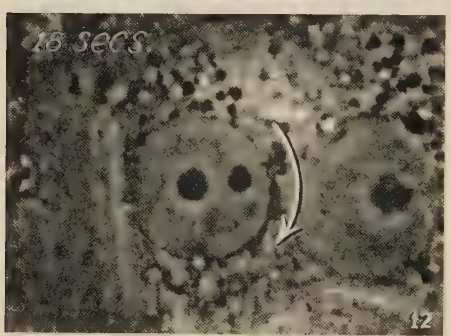
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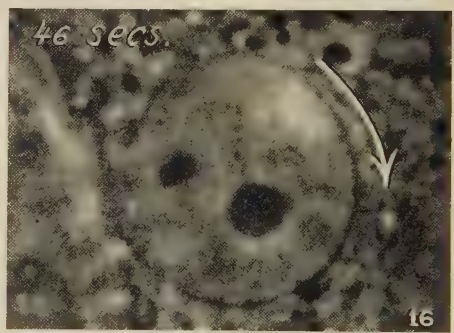
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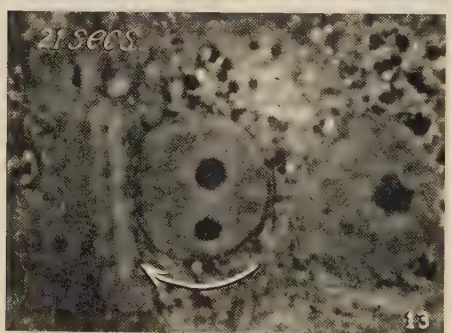
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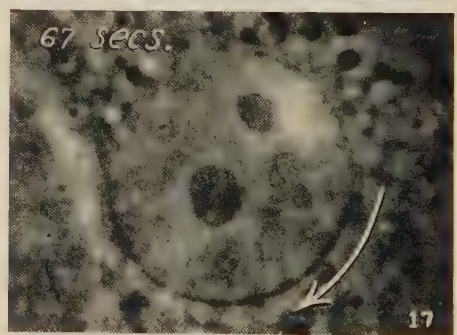
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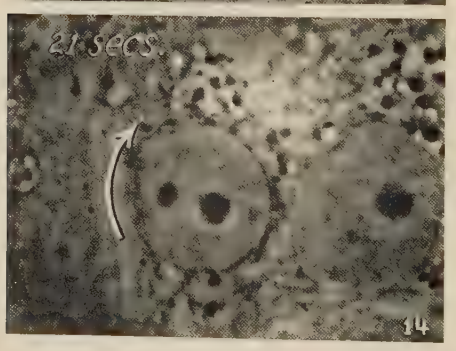
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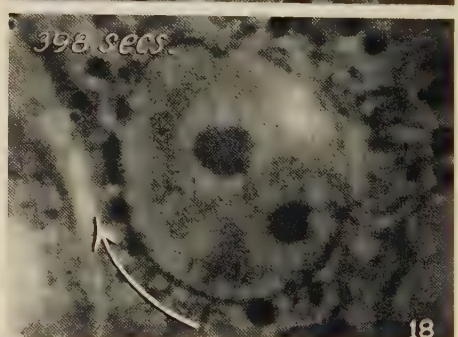
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membrane and nucleoli were drawn in pencil and then the lines were reinforced with India ink. Advantage was taken of the fact that the nuclei selected for the outline contained two nucleoli of different sizes so that a pencil line could be drawn through their centers and thus provide a means of determining the direction of rotation, using the intermediate point between the nucleoli as an "axis of rotation" (see inset in Fig. 9). By means of a protractor centered upon the axis of rotation, the direction of rotation could be read off in angular degrees. Graphs were made to describe rotation as a function of frame number and thus of time. In some fields at least 20 per cent of the cells showed nuclei rotating in both directions (Figs. 1–4). A short sequence at higher magnification was selected to illustrate shifts in the direction of rotation during a relatively short time interval. Figs. 5–8 and the graph in Fig. 9 were prepared from the same part of the film. These show that changes in the direction of rotation are relatively rapid and that movements in both clockwise and counterclockwise directions may occur twice within a 45 minute interval.

The film sequence showing the most frequent and the most rapid complete nuclear rotations found thus far in our series of records is illustrated graphically in Fig. 10. Within a period of 70 minutes the nucleus under study had completed 15 revolutions or an average of 280 seconds per revolution. Actually, at one period the nucleus described a complete revolution in 75 seconds. Enlargements from a typical portion of this record are shown in Figs. 11–14. The rate of rotation appeared to be remarkably regular. No slight slowing of speed which might indicate polarization within the nucleus correlated with cytoplasmic structures such as the juxtanuclear zone could be detected. All of the rotations in this series were in a clockwise direction.

Moving pictures made with a 1.9 mm objective and a 10 × ocular yielded records showing a nucleus which extended almost completely across the short axis of the 16 mm film frame (Figs. 15–18). Careful examination of the record did not reveal specially differentiated structures in the immediate perinuclear area or any change in optical density which might be associated with the rotation of the nucleus. However, in the last part of the sequence a small notch was seen to develop at one pole of the nuclear membrane (Fig. 18). Efforts to find other examples of such deformation have not been rewarded.

DISCUSSION

While there is a paucity of information available in the literature regarding rotational movements of nuclei except when these are associated with the preparation for spindle formation in the mitotic process, several workers

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Figs. 11–18. Enlargements from film frames of living adult human nasal mucosa in tissue culture. Phase contrast microscopy. The time lapse between successive frames is given in seconds at the upper left of each photograph. Arrows indicate the direction of movement.

Figs. 11–14. An example of a nucleus showing rapid rotation taken from the film sequence used for the preparation of the graph represented in Figure 10. Magnification approximately 1500 ×.

Figs. 15–18. A nucleus showing relatively slow rotation but studied at higher magnification in an effort to locate possible changes in the region of the nuclear membrane. The only interesting event which was found was the development of a notch in the membrane itself as seen in Figure 18. Magnification approximately 2000 ×.

employing time-lapse cinematography in connection with studies upon living cells in tissue culture have often observed at least some rocking motion of nuclei (1, 2). In our own film collection complete nuclear rotation has been found in epithelial cells from the anterior lobe of the hypophysis of a 5 month human embryo, cells whose identity has not yet been established from the spinal cord of an adult cat and from epithelial elements of several human carcinomas. The frequency and rapidity of rotating nuclei in cultures of epithelial cells derived from the adult human nasal mucosa, however, strikingly demonstrates a phenomenon which may be of fairly general occurrence, particularly in epithelial tissues under the conditions described in this study. The possibility that rapid complete rotation obtains in the intact organism remains, to our knowledge, an unexplored problem.

In the light of growing information regarding the movement of material synthesized in the nucleus across the nuclear membrane, rotatory activity of the nucleus assumes a new importance as a possible mechanism for the distribution in the cytoplasm of formed nuclear products.

Attention is being directed at determining the physico-chemical factors which may be critical in determining the rate of nuclear rotation.

Grateful acknowledgment is made to the staff of the Department of Otorhinolaryngology for cooperation in obtaining suitable biopsies of nasal mucosa.

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THE DETERMINATION OF SMALL AMOUNTS OF P^{32} LABELED DNA IN TISSUE BY CO-PRECIPITATION WITH AN INACTIVE CARRIER

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In studies of nucleic acid turnover with radioactive tracers one is sometimes faced with the problem of working with so small amounts of tissue that the common isolation methods fail to give enough material. Provided the idea is only to perform *relative* determinations of DNA turnover in a material which has been subjected to various experimental treatments, *e.g.* x-rayed *versus* non-x-rayed cells, it would seem worth while to try a co-precipitation procedure with inactive DNA as a carrier in the process of purification. This implies that the carrier and the labeled DNA mix homogenously and are precipitated in stoechiometric relations by the usual methods of purification.

A number of trials on DNA collected from various sources suggest a rather wide adaptability of this principle.

EXPERIMENTAL

It would seem preferable in this case to avoid such methods for the isolation of DNA as involve the use of rather crude procedures like treatment with strong alkali and hot TCA (2). Considering this, the inactive carrier used throughout this work was prepared from calf thymus according to Gulland (1). The essential point in the procedure is the use of 10 per cent NaCl at a low temperature (0–2°) for the extraction of the nucleoprotein. Some of the experiments reported below were performed with this nucleoprotein as a carrier. In other trials the nucleoprotein was redissolved in 10 per cent NaCl and repeatedly (6–10 times) shaken with chloroform-amyl-alcohol until protein-free (biuret test). The DNA-carrier, which was obtained after precipitation with alcohol, washing and drying over P_2O_5 may possibly be expected to be of a more uniform composition than preparations obtained according to Schmidt-Thannhauser. Finally the DNA was dialyzed in order to remove possible low molecular contaminants. With the

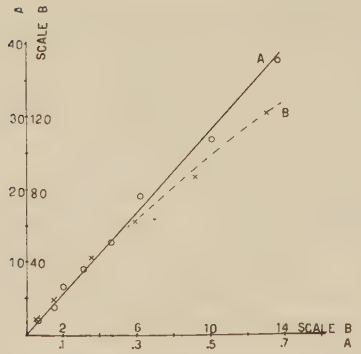

Fig. 1. Abscissa: Weight of labeled compound in mg. Ordinate: Counts/min/mg coprecipitate.

aid of these two carriers, the nucleoprotein and the purified DNA, a series of trials were made as exemplified in the following experiments.

1. *Co-precipitation of DNA from intestines of rabbit, labeled with* P^{32}. A first attempt was made to redissolve small amounts of P^{32} labeled DNA from rabbits, also prepared and purified according to Gulland, with the DNA carrier to ascertain if and to what extent the co-precipitation of two heterologous DNA's is possible.

0.03–0.7 mg of this labeled rabbit DNA was weighed in on microbalance and in each case mixed with 20 mg DNA carrier in 10 ml 10 per cent NaCl in a Turmix micro-blender. The solutions were left for 36 hours in the refrigerator at +2° and occasionally stirred to facilitate homogenization and dissolving. The viscous liquids, which contained but traces of undissolved material, were centrifuged (10 000 RPM) to give a clear solution and were precipitated with an equal volume of ice-cold ethanol; the DNA-threads were wound on a glass rod, washed free from Cl with 70 per cent alcohol, then with ethanol and ether and dried. Yield 11.3–11.5 mg. Activity measurements on the samples were performed using a lead-shielded GM-counter with a very low background (~ 2.5 imp/min) for sufficiently long periods to obtain significant results.

The results are plotted in Fig. 1 A, calculated as counts/min/mg of co-precipitated mixture of active and inactive DNA. The linear relation between the weight of labeled DNA and counts/min is satisfactory.

In another series a crude sample from the same rabbit was prepared. RNA was first extracted from the ground tissue by repeated extractions with 0.9 per cent NaCl and the solid matter was washed with alcohol and ether and dried. The mixtures of labeled compound + 10 mg nucleoprotein carrier were extracted with 10 ml 10 per cent NaCl for two days in the cold and were during that time frequently vibrated

in a mechanical stirrer, giving 5 000 vibrations/min., to facilitate dispersion. After centrifugation the undissolved rests were treated a second time over night with 3 ml 10 per cent NaCl and the combined extracts shaken until protein-free with chloroform-amylalcohol in the proportion 3.5 : 1 (6 times). The products were dialyzed in a rocking device until conductivity measurements of the outside water indicated that no further diffusion of low molecular substances took place. Finally they were reprecipitated with alcohol etc.

The results of the activity measurements of aliquot samples are plotted in Fig. 1 B. There is a good correlation between the initial weight of labeled crude DNA material and c.p.m. to some 6 mg, while higher sample weights give too low counts. Obviously the NaCl-solution approaches the saturation point here. A check of the activity in the undissolved remainder also proved this to be true. By trial it should be easy, however, to adjust the volume of NaCl-solution to allow the extraction of labeled samples within any range.

The results so far indicate the possibility of carrying out analysis of the relative amounts of labeled DNA in such small samples as are impossible or difficult to handle with ordinary isolation methods, say of the order 0.05–5 mg dry weight, provided the specific activity is sufficiently high. They also show that it would be appropriate to start the combined purification of labeled compound and carrier from a dried stage which is easy to prepare.

2. *Experiments with ascites tumour cells.* From an investigation of the action of x-rays on the incorporation of P^{32} in the DNA of Ehrlich mouse ascites tumour cells, the following experiments may be cited in which the ordinary isolation and purification method may be compared with the carrier procedure.

One group of 8 mice which had been grafted with the tumour were given an irradiation of 2 000 r and immediately afterwards a tracer dose of P^{32}. They were killed 2 hours later. One control group was treated likewise, excepting the irradiation. The c.p.m./mg DNA in x-rayed and control groups of animals as computed from preparations obtained with the ordinary macromethod indicated a depression caused by the irradiation amounting to −18.7 per cent.

In parallel experiments part of the fresh ascites samples were after extraction with 0.9 per cent NaCl, washing and drying weighed in and run through the procedure with the nucleoprotein carrier.

From an average of duplicate experiments in which around 0.5–2 mg dried cell material was used −15.4 per cent depression was established (Table I). This is in fairly good agreement with the figure above. In general, when higher accuracy is wanted, it would be advisable to run the experiments in triplicate or more.

Table I

Groups	mg dried ascites sample	c.p.m./3 mg DNA mixture	Percentage decrease in x-rayed sample
2 000 r	0.49	5.2	−16.1
control	0.49	6.2	mean = −15.4
2 000 r	2.13	21.5	−14.7
control	2.13	25.2	

3. *Experiments with root tips of Allium cepa.* In a third series of experiments the same principle was applied for a study of the P^{32} incorporation into plant material, namely the meristem zone of the root tips of *Allium cepa*, which contain cells in rapid mitosis. Without a very large supply of material it is impossible to isolate the DNA in ordinary ways—the wet weight of a single tip is about 0.4–0.5 mg—but the carrier method gives a possibility to perform relative comparisons between different root samples. The following experiment was one of several methodological trials and shows the degree of accuracy.

A number of root tips from onions grown in a P^{32} containing solution, was treated as stated above, giving a dry preparation of cell nuclei and debris. Of this preparation the amounts given in Table II were mixed with 6 mg thymonucleoprotein in 6 ml 10 per cent NaCl and subjected to the purification procedure including chloroform-amylalcohol treatment and dialysis.

Table II states the activities of 3 mg of the mixture of carrier + unknown DNA as well as these figures divided by the corresponding weights of the active root tip material. This last column shows that a determination of the turnover per unit weight of root-material, based on a mean of several, say, five analyses may be expected to give a good estimate. The method was worked out to be used for a study of the influence of x-rays on the DNA turnover in the meristem zone of the roots.

Table II

mg dried root tips	counts/min./3 mg mixed DNA	counts/min./mg root tips
0.27	6.0	22.2
0.63	18.1	28.7
1.01	21.0	20.6 mean 23.9 ± 1.6
1.46	38.9	26.6
1.99	41.8	21.0

CONCLUSIONS

It would appear from the examples given above that the method outlined might be useful for estimations of the relative turnover of DNA from various sources and in small amounts provided the problem does not involve further analysis of the purine and pyrimidine components. In such cases methods of isotope dilution and/or chromatography should be used.

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FURTHER OBSERVATIONS ON THE STRUCTURE OF MYELIN

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The effects of drying, temperature changes, and treatment with lipid solvents on the structure of the myelin sheath of frog sciatic nerve have been studied by X-ray diffraction methods (2, 3, 4), and preliminary conclusions as to the type of structure present have been made (9).

Earlier investigators (12, 13, 14) had suggested that myelin was a lipoprotein structure, and our X-ray diffraction results are in keeping with this suggestion, though the structure might be better described as lipid-lipoprotein, for experimental modifications of the structure (2, 3, 4) have indicated that there is a labile lipid component which can be readily separated from the original structural unit leaving a 'residual complex' containing more tightly bound lipid molecules. Schmitt, Bear, and Palmer (14) proposed several schemes whereby a fresh nerve fundamental unit of 171 Å could be built up from bimolecular leaflets of lipid and thin layers of protein. Further considerations of the X-ray diffraction data and the molecular structures of individual lipid components have indicated possible ways in which a more precise picture for myelin structure might be obtained. The purpose of this communication is to attempt a more detailed interpretation of some of the X-ray diffraction data published previously, and thereby to suggest new features of myelin structure.

Low angle diffraction patterns of fresh sciatic nerve of frog and normal dried nerve

The low angle pattern of fresh nerve includes the five bands shown in Fig. 1, and possibly higher spacings which have not yet been resolved. These long spacing bands all show the same precise equatorial orientation when the structure is examined perpendicular to the fibre direction, and can be accounted for as different orders of diffraction of one fundamental repeating unit which is orientated radially in the myelin sheath. This repeating unit must have a length of about 171 Å or some simple multiple of this. The

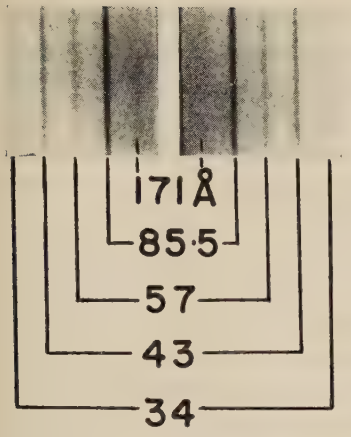

Fig. 1.

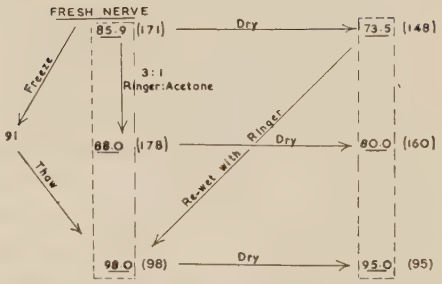

Fig. 2.

Fig. 1. Low-angle diffraction pattern of fresh frog sciatic nerve (Slit collimating system).
Fig. 2. Diagram summarizing the effects on X-ray diffractions spacings of some modifications of nerve structure.

alternation of intensities of the orders of diffraction, and the fact that the probable first order (171 Å) is a very weak diffraction, suggest that this unit contains two very similar sub-units which have almost identical scattering powers for X-rays. They may differ slightly in composition or in geometrical arrangement or both.

It has been shown (2, 3) that the long spacing diffraction bands of dried nerve can be divided into two groups. The three lower spacings at approximately 34.5 Å, 43 Å, and 60 Å appear to be associated with lipid materials which have separated from the main structure leaving a 'residual complex' which is responsible for the two higher spacings at approximately 73.5 Å and 148 Å. The second order diffraction of the residual complex is also much more intense than the first order, again indicating a tendency for 'halving' to occur.

Electron microscope photographs of nerve preparations (5–8, 15) have shown that the myelin sheath has a concentric layered structure, but give a value for the repeating unit in the radial direction which is about one half that to be expected from X-ray diffraction data. Thus the 148 Å spacing of the residual complex of normal dried nerve, and probably also the 171 Å spacing of fresh nerve, represent the thickness of two such layers which differ only slightly in geometry or composition so as to appear identical in

the electron microscope. It is, however, also possible that the methods for the preparation of the electron microscope specimens eliminate the slight difference between alternate layers which is demonstrated in the diffraction patterns of fresh nerve.

The effects of modifications of structure on the low angle diffraction patterns

When the principal spacings in the diffraction pattern of fresh normal nerve are compared with those of specimens which have been modified either by previous drying (2), or by freezing or heating to about 60° C (3), or by treatment with lipid solvents (4), and then re-wet with Ringer solution at room temperature, it is seen that the modifications apparently lead to increases in spacing. Similarly, when 'residual complex' spacings of normal dried nerve are compared with those of nerve which has been modified before drying, it is again found that the modified specimens show the higher spacings. Examples of such spacings are given in Fig. 2. The main spacing quoted is that of the most intense diffraction band in the pattern, whilst the figure given in brackets is the probable fundamental spacing.

The changes in spacings brought about by these modifications would appear to reflect a variation in layer thickness within the structure. In addition, the fact that modifications cause a disappearance of the odd orders of diffraction in the wet nerve patterns and make the first order diffraction of the residual complex unit in dried specimens increasingly difficult to detect, suggests that the expansion of the structure tends to make the two halves of the original structural unit more nearly identical so that the strongest diffraction band (i.e. the one in the region of 90 to 100 Å) becomes the fundamental.

Whilst the variations in the spacings of wet specimens may be associated with the thickness of water layers in the structure, the changes in the residual complex spacings of dried material must be accounted for in terms of changes in thickness of lipid layers or of protein layers or of both. Little is known concerning the nature of the protein component of myelin so it is difficult to predict what part it may play in the apparent expansion of the residual complex. However, a consideration of structures consisting of alternating lipid layers and protein layers such as were suggested for fresh myelin by Schmitt, Bear, and Palmer (14), indicates a type of modification which may account for some of the experimental observations. If from such a structure every second layer of lipid is eliminated, then the modified unit will include two layers of protein for every lipid layer. This might result in an increase in spacing

of the strongest diffraction band by an amount corresponding to the thickness of one protein layer, and at the same time the necessity for including two such sub-units in the fundamental repeating unit might be eliminated. This possibility is illustrated diagrammatically in Fig. 3. In this schematic drawing of the repeating unit of the normal residual complex, bimolecular leaflets of lipid alternate with thin layers of protein. The necessary difference between the two halves of the unit is illustrated by having the lipid molecules assume different orientations towards the protein layers. In such an arrangement the strengths of association of lipid and protein in the two layers would be expected to differ, and the elimination of alternate layers of lipid under certain conditions might be readily understood.

This type of modification would, however, be inflexible. One would expect a single definite expansion corresponding to the thickness of a protein layer, or possibly a series of such steps, whereas the experimental evidence points to a continuous variation of the spacings with a gradual weakening of the first order diffraction above about 180 Å. Furthermore, the elimination of 50 per cent of the lipid from the residual complex with each modification should be reflected in a corresponding increase in the intensities of the 'free lipid' bands in the diffraction pattern. In general the X-ray diffraction data indicate that the most extensive liberation of lipid occurs during normal drying, and that further modifications such as the ones indicated in Fig. 2 are accompanied by relatively small separations of lipid. A third criticism is that such a structure as the one illustrated in Fig. 3 does not readily lead to an understanding of the detailed changes which are observed during normal slow drying.

A much more satisfactory explanation of modifications of nerve myelin in general can be obtained by considering changes in the organization of the lipid layers. In the case of the apparent expansion of the residual complex which accompanies modification of myelin, the actual magnitude of the expansion might be accounted for in terms of variations in the angles of tilt of the long axes of the molecules in the lipid leaflets. It has been noted previously (10) that isolated phospholipids can exist in several polymorphic forms in which the thickness of the bimolecular leaflets seems to vary. This variation is almost certainly due to changes in the angles of tilt of the long lipid molecules with respect to the plane of the bimolecular leaflet. Polymorphism has also been demonstrated among the nerve lipids (3). The spacings at 43.5 Å and 60 Å in the patterns of total lipid extract and of dried nerve appear to be readily interconvertible by appropriate changes in the temperature of the specimen. When total lipid extract was fractionated, some

of the fractions showed even greater changes in spacing with temperature. The fraction which gave the highest spacing of about 65 Å at 20° C showed a spacing of less than 40 Å at 100° C. All these changes appear to be accounted for by changes in the angle of tilt of the lipid molecules within the bimolecular leaflets. Thus the changes in the residual complex spacings in dried nerve may reflect changes in the angle of tilt of molecules in the lipid layers, though causes other than temperature changes must be operating. These changes in the lipid layers may be accompanied by or even result from a separation of some of the shorter chain lipids from the complex. Also, it has been noted previously that the conditions under which modification of the complex took place were such as to suggest that a protein component was being affected. Thus it may be that the organization of the lipid layers is stabilized by the association with protein, and that modification of the protein leads to re-organization of the lipid layers. This would be in keeping with the irreversibility of most of the modifications of the complex structure, and would allow for intermediate stages of re-organization as the protein were progressively modified.

The differences in the organization of alternate bimolecular leaflets of lipid might still be due to different orientations towards the protein layer as was suggested in Fig. 3. A more simple picture would be one in which the differences were due to variations in directions of tilt of the lipid molecules. The tilting may be such as to make adjacent layers approximately mirror images of each other. This would allow for the inclusion of two bimolecular leaflets of lipid in the unit cell until the angle of tilt decreased to zero (i.e. until the lipid molecules contributed their fully extended length to the thickness of the leaflet). Adjacent layers would then presumably become identical, and the necessity for including two bimolecular leaflets of lipid in the unit cell would disappear. This would account for the fact that modified residual complex spacings in dried specimens of 95 to 100 Å appear to be the fundamental spacings (i.e. no higher spacings are recorded), whereas the residual complex spacings of 73.5 Å to 90 Å are probably second order, a fainter first order band being detected in the region 140 to 180 Å.

If the spacings in the region of 95 to 100 Å represent a structural unit containing one bimolecular leaflet of lipid in which the long axes of the lipid molecules are perpendicular to the plane of the leaflet, it may be of interest to consider the dimensions of such a leaflet. In the mixed lipid specimen of total lipid extract, the highest spacing is about 60 Å, and if this is the thickness of the lipid layer in the modified residual complex then a possible thickness of 35 Å or more remains for the protein layer. It may be that at

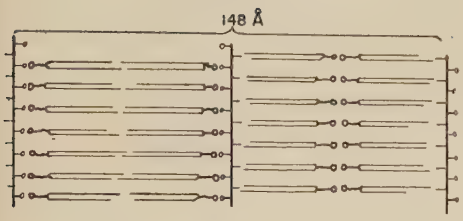

Fig. 3.

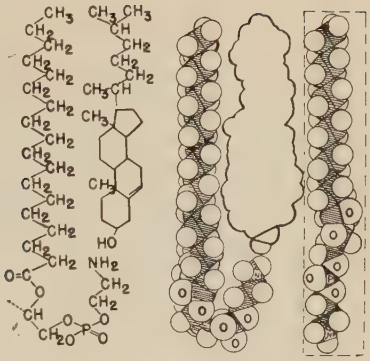

Fig. 4.

Fig. 3. Schematic drawing of a type of 'residual complex' structure which fulfils some of the X-ray diffraction requirements.
Fig. 4. Drawing illustrating a possible type of cholesterol-phospholipid complex in normal fresh nerve.

this stage of the modifications appreciable fractionation of the lipids has already taken place, and only the longest molecules remain in the residual complex. Even so, the highest spacing observed in fractions of nerve lipid is of the order of 65 Å, and if this figure is considered to apply to the modified residual complex there still remains a possible thickness of about 30 Å for the protein layer. It is, of course, possible that the myelin contains a lipid complex of dimensions greater than 65 Å and that this complex is broken down during extraction, but on the present evidence there would seem to be a definite possibility that the protein layer is about 30 Å thick.

If the protein layer of the residual complex of normal dried nerve is also considered to be about 30 Å thick, then the lipid leaflet here would have a thickness of about 43 Å (which coincides with one of the total lipid extract spacings), and would presumably contain lipids in a highly tilted form.

These ideas on the kind of molecular structure indicated for the residual complex of normal dried nerve and its modifications are represented diagrammatically in Fig. 5. The representation of the lipid molecules of the normal dried nerve complex as being curled as well as tilted is in line with a previous publication on the structure of pure phospholipids (10) in which it was suggested that the curling of the end group of the phospholipid molecule into a position of more stable orientation with its neighbour was the actual reason for the tilting of the long axis of the molecule with respect to the plane of the leaflet.

Suggestion of new structural units for fresh myelin

If the spacings of the fresh nerve diffraction pattern are compared with those of modified dried nerve, and the thickness of the protein layers assumed to be approximately the same for both structures, then it is clear that in fresh nerve too the longer lipid molecules must be curled or tilted in some way in order to accommodate them in the layers. Considering the protein layer to be approximately 30 Å thick, and the fundamental unit of 171 Å to contain two layers of lipid and two of protein, the spacing available for each bimolecular leaflet of lipid would be at most 55 Å, and probably less for there may also be layers of water in such a structure. The lipid molecules may be tilted as in the case of the residual complex of normal dried nerve, but in a fluid structure such as fresh myelin where the molecules are probably relatively widely spaced it is difficult to visualise a stable tilted arrangement of molecules. Furthermore, the polarized light observations on fresh nerve indicate a radial orientation of structural units such as would allow only a very small degree of tilting. The alternative picture of curled or bent molecules would appear to require some scheme whereby the long molecules could be stabilized individually in a curled position. In seeking some kind of stabilizing mechanism the presence in myelin of large amounts of free cholesterol has also been considered, and it would seem that there is a possibility of complex formation between a cholesterol molecule and a long chain lipid with a polar end group such as a phospholipid. There have been indications from other sources that cholesterol and phospholipid, particulary cephalin (11), will form a stable complex, and a consideration of the individual molecular structures leads to the suggestion illustrated in Fig. 4. Cholesterol is present in myelin in approximately equimolecular proportions with the total phospholipid (1), so that a stabilization of the longest lipid molecules individually by cholesterol molecules would seem possible. Furthermore, it is probably significant that only free cholesterol is present in myelin, and this suggests that the —OH group is involved in the complex formation.

In the complex illustrated in Fig. 4 the end group of the phospholipid molecule is curled around so that it points back along the hydrocarbon chain in a manner very similar to that postulated for the highly tilted polymorph of the pure phospholipid (10). Models suggest that such a curling would be easily accomplished. The hydrocarbon part of the cholesterol molecule lies alongside the hydrocarbon chain of the phospholipid so that the non-polar ends of both molecules are in line. The hydroxyl group of the cholesterol is then close to the terminal group of the phospholipid so that some polar

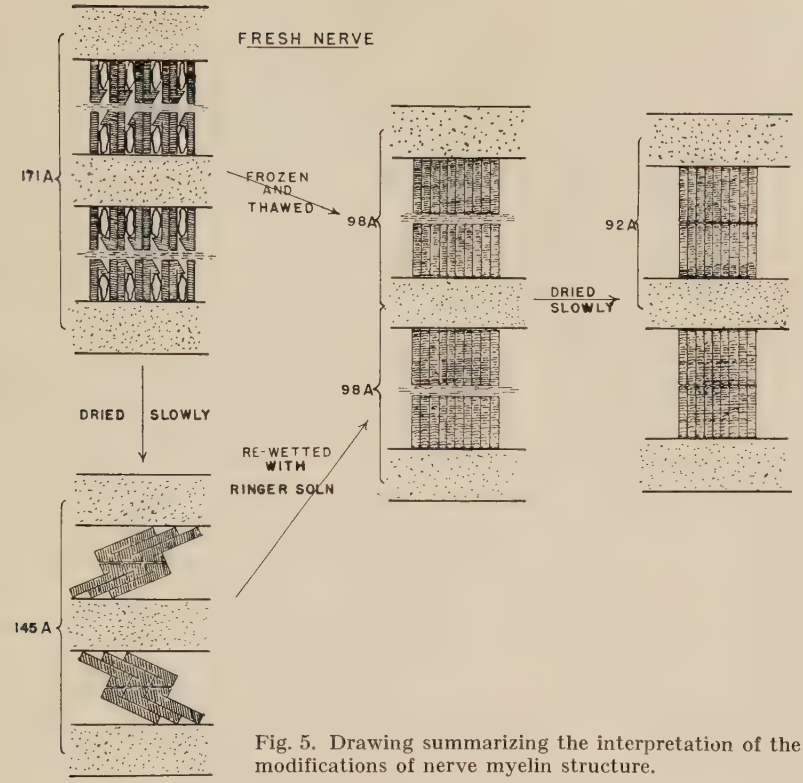

Fig. 5. Drawing summarizing the interpretation of the modifications of nerve myelin structure.

interaction should be possible. In the case of the phosphatidyl ethanolamine molecule used in the illustration there would appear to be the possibility of hydrogen bond formation, and presumably there would be the same possibility with serine, though in this case there would be an additional carboxyl group to be considered in the association. With many of the lipid components of myelin such a strong linkage as is given by a hydrogen bond could not be achieved, but nevertheless some kind of polar interaction might be possible between the end groups giving complexes of varying stability. The complex would thus be stabilized by the van der Waal's association between the hydrocarbon portions of the molecule and the polar interaction of the end groups, and would serve to accommodate the longest and the shortest molecules in individual stable units in a layer of restricted thickness.

If the fully extended lengths of the longest lipid molecules in myelin are

of the order of 65 Å then in such an association with cholesterol as has been suggested, the length should be effectively reduced to about 50 Å. The lengths of these complex units may determine the thickness of the lipid layers, and other molecules may be accommodated in the fully extended form as illustrated in Fig. 4. With this thickness of lipid layer in the structure of fresh myelin the water layer would appear to be very thin (i.e. of the order of 5 Å). This would be quite in keeping with the strict regularity of the layer spacings as indicated by the sharpness of the bands in the X-ray diffraction pattern, though provision must be made somewhere in the structure for the large amounts of water known to be present.

A structural unit for fresh myelin, incorporating the cholesterol-phospholipid (or cerebroside) complex and these proposed layer thicknesses, is illustrated in Fig. 5. An additional fully extended lipid molecule (as in Fig. 4) is also included to indicate that such molecules may be associated with the complex units to give an extensive structural organization within the lipid layers. The complex unit is asymmetric about the long axis and it is possible that any larger structural units involving this complex may also be asymmetrical. Thus one of the simplest ways of representing the necessary difference in geometrical arrangement between the two lipid bimolecular leaflets in the structural repeating unit would be to make them approximate to mirror images of each other across the plane of the protein layer as illustrated. Such an arrangement would place limitations on the free rotation of the complex units about their long axes, and would in fact infer a definite organization of molecules within the lipid layer.

Effects of these new suggestions for myelin structure on detailed interpretation of normal drying changes

The effect that the introduction of this new complex unit into the structure of myelin has on the interpretation of the detailed changes in diffraction pattern observed during slow drying (2) must now be considered. If the long lipid molecules in the complex are to form a highly tilted structure such as has been proposed in the case of normal dried nerve, it would probably be necessary to break the association with the cholesterol. In fact the tilted structure would appear to be more easily formed with a uniform group of molecules. However, although a considerable amount of lipid separates from the complex during slow drying there is no evidence for preferential separation of lipids of certain types. The relative intensities of the free lipid bands in the normal dried nerve pattern are similar to those of the diffrac-

tion pattern of total lipid extract. Thus, although all complex formation may be broken down, not all of the cholesterol can be expected to be expelled from the residual complex. The double layer of cholesterol molecules can of course be accommodated in the 43 Å lipid layer of normal dried nerve without tilting, and it is possible that this layer presents a mosaic pattern containing separate phases or patches of lipid, in which the long axes of the molecules are tilted at different angles to the plane of the layer. The kind of structural arrangement for the residual complex of normal dried nerve which is shown in Fig. 5 may only represent a part of the mosaic pattern, namely that occupied by the longest molecules, but it would seem probable that the arrangement of these longer molecules again determines the thickness of the leaflet. There are several possible reasons for the formation of a highly tilted structure at this stage of modification of the complex. It may be, as has been suggested previously, that it results from the nature of the association of the lipid with the protein, and that it is only after further modification of the protein that the long lipid molecules can assume the less tilted arrangement which appears to be the more stable form at room temperature. Alternatively it may be due to the continued presence in the lipid layer of the shorter lipid molecules which, if they are to be accommodated independantly, put certain limitations on the layer thickness which may necessitate a tilting of the longer molecules. Subsequent modifications of residual complex may result in the gradual elimination of the shorter lipids, thus allowing the long ones to assume a more upright position. This effect may in fact be consequent upon the first, for the continued presence in the complex of the shorter molecules may be governed by their association with the protein. In general it would appear that the increase in intensity of the 57 to 60 Å diffraction band during the first stage of drying of fresh nerve reflects the formation of a separate lipid phase containing both long and short molecules. This separation of lipid may be due to a breakdown of some of the associations between phospholipids or cerebrosides and cholesterol which results in the expulsion of both molecules from the lipoprotein structure. After dissociation from the complex, the longer lipid molecules can presumably become fully extended to give a bimolecular layer thickness of about 60 Å. The shorter molecules can be accommodated in this layer until the later stages of drying when three separate phases of lipid are formed. Thus, although both long and short lipid molecules are separated from the main structure during drying, and the X-ray diffraction patterns show no evidence for preferential separation of lipids according to chain length, there may still be a preferential separation according to the strength of association

both in lipid complex formation and in attachment of the lipid complex to the protein. There is, however, no ready explanation of the apparent reversibility of the initial stages of the modifications. During stage 2 (2) of slow drying, the diffraction data show no further separation of lipid, but indicates an extensive reorganization within the residual complex. The decrease of the residual complex spacing from about 85 Å to about 74 Å is now assumed to reflect both a disappearance of water layers and a tilting of the long chain lipids.

Wide angle diffraction patterns of nerve

As pointed out previously, if the structure of fresh myelin approximates to that illustrated in Fig. 5, there must be a definite organization of molecules within the lipid layer. Such an organization might be expected to be reflected in the appearance of the wide angle pattern.

The 4.7 Å spacing, and the faint 9.4 Å spacing reported by Schmitt, Bear, and Clark (13), show marked meridional intensifications, but this can be at least partly explained in terms of the radial arrangement of the lipid molecules (13). There is also the equatorial 15 Å diffraction which because of its sharp appearance and the direction of its orientation has been considered to be associated with the layer spacings in the radial direction, possibly as a high order (11th) of the fundamental long spacing. However, there are certain features of the changes observed in this band when the structure is modified, which are not easily related to the behaviour of the higher spacings. Thus, for instance, on freezing, the 15 Å spacing appears to increase to about 19.6 Å, and to become less well orientated. This increase is much greater than would be expected from a comparison with the expansion of the higher spacings, and the loss in orientation would seem to associate it more with the wide angle diffraction than with the long spacings. The orientation of this 15 Å band could be related to an orderly arrangement within the lipid layer and still perpendicular to the fibre direction, but this would necessitate a precise crystalline array within the lipid layer giving a 4.7 Å or 9.4 Å repeating unit in the fibre direction, and a 15 Å spacing at right angles to this. A more detailed study of the wide angle pattern of nerve, possibly using a single nerve fibre and micro-camera would appear to be necessary to decide whether this is a serious possibility.

In such a study, the possible contribution of a protein component to the wide angle diffraction would also have to be considered. In the radial direction, the diffracting unit is a composite lipoprotein structure, and any characteristic protein spacing in this direction would not produce diffraction be-

cause the protein structure is not continuous. However, in directions in which the protein layers are thought to be continuous, namely in directions perpendicular to the long spacings, characteristic protein diffraction might be expected. As has been pointed out previously, the protein component might contribute to any of the meridionally accentuated wide angle spacings and even to the equatorially orientated diffraction at about 15 Å. However, lipid extraction experiments have shown that the greatest contribution to the 4.7 Å diffraction band comes from lipid molecules, and the changes induced in the 15 Å equatorial spacing by various modifications of structure indicate that this too is probably associated with lipid. The faint meridionally accentuated 2.8 Å spacing which is seen in some patterns may be a protein diffraction, but the fact that the diffraction pattern comes from the whole sciatic bundle makes it impossible to decide whether this could reflect a protein component of myelin. In general it would seem that the lipid and lipoprotein diffractions are predominant in the pattern, and the contribution of characteristic protein diffraction is relatively small.

CONCLUSIONS

The introduction into the myelin picture of a complex structural unit involving the stabilization of a long-chain polar lipid molecule in a curled form by association with a cholesterol molecule, can be included in a detailed interpretation of most of the X-ray diffraction data. The picture is by no means complete, and the suggestions are only a few of the structural features which may be included in myelin architecture. Further details of the picture can probably be derived from a careful consideration of the structures of each individual myelin component when these have been more definitely characterized by chemical methods.

The geometrical arrangement of these complexes and other lipid molecules in the lipid layers cannot definitely be established on the present experimental evidence. It seems likely, however, that there is a slight difference in geometrical arrangement in alternate layers, and, in case of the residual complex in the dried specimens, to represent this difference as a variation of the direction of tilt of the long axes of the lipid molecules gives a ready explanation of the elimination of the difference when the structure is expanded and the lipid molecules assume an upright position. In such a case it is not necessary to assume any difference in the nature of the association of the lipid molecules with the protein layers, and in the drawings this has been made uniform, with the hydrocarbon portions of the lipid always ori-

entated towards the protein layer. However, on present evidence the picture given for both fresh myelin and residual complex might equally well be reversed so that the polar portions of the lipid molecules are orientated towards the protein layers.

In the case of the fresh myelin unit, the direction of orientation of the polar portions of the lipid molecules is also assumed to be uniform in all layers, and in representing the geometrical differences between alternate layers in this structure, use is made of the asymmetry of the complex unit, and adjacent layers are made mirror images of each other. This can only be the case if there is a definite ordering of the lipid molecules within the plane of the layers, and the odd orders of diffraction of the long spacing should disappear if the complexes are broken down or if the organization is disturbed so that rotation of the complexes about their long axes becomes possible. Confirmation of this type of arrangement might be obtained by a precise comparison of the changes in intensities and degrees of orientation of long and short spacing diffractions which accompany slight modifications of structure. If the structure approaches the ideal illustrated, it is possible that some slight experimental modification might be obtained which destroys the organization of lipid within the layers without affecting the spacing of the layers in the radial direction. In terms of the structure shown above, this should result in the odd orders of long spacing diffraction becoming much weaker and the wide angle diffractions losing their orientation. Some indications of such changes have already been observed in experiments in which nerve has been heated to 40° to 50° C for a short time. The changes are, however, relatively small, and are soon complicated by changes in long spacings which indicate that the structure is breaking down completely.

In addition to the differences in geometrical arrangement of lipid in adjacent layers, there may also be a small difference in composition. Preliminary studies on the effects of osmic acid fixation on the structure of myelin show that the deposition of osmium in the layers tends to intensify the first order diffraction, thus indicating a difference in the density of deposition in the two layers. The osmic acid also tends to bind the lipid more firmly in the layered structure so that further modifications are not readily produced. These experiments may give more information on the nature of the association of the lipid with the protein layers which are assumed to be present.

In general, the background of information already obtained seems to warrant a continuation of this type of study on more complex changes such as is already being undertaken in studying the effects of fixation, and also on changes of a more physiological nature such as degeneration, in which the

initial structural modifications seem to be of a more subtle nature, involving primarily changes in relative intensities of diffraction bands and their orientations. At the same time further consideration of the structures of individual components should lead to a more detailed molecular picture for fresh nerve myelin.

SUMMARY

The changes in X-ray diffraction pattern which accompany modifications of the myelin sheath of frog sciatic nerve (details of which have been published previously) have been reconsidered in detail.

It is noted that some modifications lead to increases in the diffraction spacings of the complex structure, and in the case of the dried specimens this is explained in terms of variations in the angle of tilt of lipid molecules within the layered lipoprotein structure. Approximate layer dimensions are deduced from the variation of the diffraction spacings of modified nerve, and the interpretation summarized diagrammatically.

In the case of fresh nerve myelin the necessary condensation of the lipid layer thicknesses is achieved by having the longest lipid molecules stabilized individually in a curled position by association with cholesterol molecules rather than by tilting them. This enables the myelin components to retain their radial orientation, and suggests a specific function for the free cholesterol in myelin structure.

I wish to express my gratitude to Professor A. C. Frazer and Professor J. Elkes for their continued interest and encouragement in this work.

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EFFECT OF VERSENE ON SEA URCHIN EGGS

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Versene (ethylenediamine tetraacetic acid), a chelating substance, was found by Dr. A. Tyler (personal communication) to considerably prolong the life span of the sea urchin sperm (*Lytechinus*) and to improve its reactions in the fertilization process. The effect is completely analogous to that of glycine and a number of other amino acids (8, 9). Cleland (2) observed that versene was very active in removing traces of metal, especially zinc, from sea water, which had become toxic to the normal fertilization processes of sea urchin eggs (*Psammechinus*) through metal contamination from the laboratory sea water pipelines. The elevation of the fertilization membrane of the egg was faulty in the contaminated sea water, but perfectly normal in versene treated water. The effects resembled the ripening process of sea urchin eggs described by Runnström and co-workers (4, 7, 11).

Although the promoting effect of versene on the motility and life span of the sperm was thus established, it was still not known whether versene had an additional effect on the egg. In the present investigation it is shown that versene also causes pronounced alterations in the egg.

MATERIAL

Eggs and sperms from the sea urchin *Psammechinus miliaris* were used at the Kristineberg Zoological Station, Swedish West Coast. The material occurs in two phenotypic varieties: one (the *Z*-form) is the trivial littoral form, the other (the S-form) is found at greater depths. The optimal salinities for larval development are 27 and 32–33 promille respectively (1).

Versene and glycine were dissolved in sea water of appropriate salinity, and the pH corrected to 8.0–8.1 (neutralization with 0.2 M NaOH in sea water). All sea water was collected in glass apparatus directly at the respective animal localities; sea water from the laboratory pipelines was strictly avoided.

OBSERVATIONS

Fertilizability: The egg may still be unfertilizable though full nuclear maturity has been reached. This is due to a delayed cytoplasmic maturity.

In border cases, when full maturity is still not completely reached, a low percentage of fertilization may be obtained, but the fertilization membranes are low and often one-sided, deformed or otherwise incomplete (4, 7, 11). In underripe material, glycine and other amino acids, albumin, serum etc. were found to considerably increase the fertilizability, and to cause a normal fertilization membrane to be elevated (7, 11, 14). Similar properties have been reported for other sea urchins; for review cf. (5, 9). Furthermore, it was found (9) that glycine treatment of the sperm alone is equally effective in increasing the fertilizability in underripe egg material.

If underripe egg material (Z- or S-forms) is treated with versene a completely analogous effect is observed:

Medium	Fertilization per cent	Fertilization membranes
Sea water	0–10	Low and deformed, if any.
10^{-5} M versene in sea water	25–75	Often low, sometimes normal.
10^{-4} M versene in sea water	75–100	In most cases normal.
10^{-3} M versene in sea water	90–100	Normal (high, and rapidly elevated).
1.33×10^{-3} M glycine in sea water	90–100	Normal.

At the end of the season the eggs become overripe, and are again unfertilizable. They respond in a characteristic way to treatment in hypertonic sea water (7). In such material (Z-form) versene (10^{-4} to 10^{-3} M) was found to restore fertilizability.

The promoting effect of amino acids and other metal complex formers on the fertilizability of sea urchin eggs is by no means a simple phenomenon; the substances influence the properties of both sperm and egg (sperm life span and metabolism (8, 9, 10, 12)); jelly coat swelling, egg structure and fertilizability (5, 6, 7). It is likely that glycine and other amino acids, and versene and other chelating substances all act by virtue of their metal complex forming ability. Whether the effect is chiefly on heavy metals occurring in trace concentrations, or if interactions with Ca and Mg have also to be considered, still remains to be ascertained. In any case, however, only substances occurring in the sea-water or accumulated in the living cell are concerned; a heavy metal contamination from outside sources has been ruled out through the experimental procedure. It may also be recalled here that diethyldithiocarbamate counteracts the toxic effects of Cu^{2+}, but has in itself an inhibiting effect on sperm metabolism and life span (3, 13).

Structure of the eggs. The ripe egg is stretched under the influence of the centrifugal field in the microscope centrifuge when resting in the border zone between sea water and an isotonic cane sugar solution. It is observed that 10^{-4} M versene most strikingly furthers the elongation. As will be published presently, similar results were found by Dr. G. Kriszat using albumin, certain amino acids, among which were glycine, and calcium free sea water (cf. also (6)). The common cause is to be sought in a loosening of intermolecular bonds of the cortex structure. Versene is apt to remove interlinking cations form the cortex, presumably Ca^{2+} and Mg^{2+}, and also supposed to split hydrogen bonds. The centrifugation experiments indicate that versene is also affecting the cytoplasm of the cell interior, diminishing its viscosity. (The centrifugation experiments were kindly performed by Dr. G. Kriszat.)

Unfertilized eggs are mostly slightly anisodiametric. At fertilization, structure alterations occur in the cortex and the egg becomes nearly isodiametric. On versene treatment (10^{-4} or 10^{-3} M), even unfertilized eggs are rapidly rounded off, presumably through a loosening effect on intermolecular bonds of the cortex, leading to a decreased rigidity.

Development. Glycine and certain other amino acids inhibit the development of fertilized sea urchin eggs (14). Thus 1.33×10^{-3} M glycine strongly affects the cleavage time, and cytolysis is very pronounced in the blastula and gastrula stages. The plutei are, if ever developed, very abnormal. For undisturbed development the eggs have to be transferred to normal sea water immediately after fertilization. Albumin is free from these toxic effects, and so is versene up to a concentration of 3×10^{-4} M. Only in 10^{-3} M versene are the plutei sometimes abnormal. In these cases there is mostly a depression of the arm development; only rarely does cytolysis occur.

The jelly coat. The loosening effect of versene on intermolecular bonds is also demonstrated on the size of the jelly coat. The substance causes a pronounced swelling of the jelly. This process takes effect very rapidly, and reaches a maximum at 20–30 mins. after the beginning of the treatment. Thus, in *Psammechinus* (S-form) egg material, in which the untreated egg had an average volume of $5.3 \times 10^{-}$ µl, and the jelly coat one of 11.1×10^{-4} µl, the jelly coat volume was at maximal swelling found to be 12.5×10^{-4} µl in 10^{-5} M versene, 58.6×10^{-4} µl in 10^{-4} M, and 87.1×10^{-4} µl in 10^{-3} M. The volume of the egg itself is practically unaffected by the versene treatment. On prolonged treatment (over 30 mins.), the diameter of the jelly coat gradually decreases. This is probably due to a solvation of the outer layers of the expanded jelly. Glycine and other amino acids are also known to cause a swelling of the jelly coat, as does a lowering of the pH (cf. 5).

DISCUSSION

Amino acids, peptides and proteins act on the fertilization processes of the sea urchin egg and of the eggs of a number of other animals (5, 9, 11, 14), in a manner similar to that of the metal-complex-forming substance versene (cf. *Fertilizability* above). The common property of all these agents is presumably their ability to bind and remove metal groups situated in the cortical and interior cytoplasm of the gametes, thus loosening intermolecular bonds. Whether the chief action is on trace amounts of heavy metals, as in the detoxication of zinc-contaminated sea water (2), or if Ca or Mg are the metals normally concerned, is an open question.

The promoting effect on the life span and metabolism of the sperm is very pronounced (8, 9, 10, 12). It has also been reported (9) that it is sufficient to treat the sperm alone to secure increased fertilizability. On the other hand, there are also pronounced effects on the egg itself (5, 14, and this investigation), manifested in alterations in the structure of the cortex and the egg interior; the jelly coat is also subject to colloidal changes. Whether the effects on the sperm or those on the egg are the really essential ones for the increased fertilizability, or if both kinds contribute to the improved result, cannot yet be decided. Glycine treatment of the egg alone was, however, not found sufficient (9) to improve fertilization. It may, nevertheless, be possible that the combined treatment of sperm and egg is the most effective.

SUMMARY

Versene (ethylenediamine tetraacetic acid) increases the fertilizability of underripe and overripe sea urchin eggs (*Psammechinus*). It decreases the viscosity of the cortex and interior cytoplasm of the egg and causes the jelly coat to swell. It does not inhibit the development of the larvae.

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STRUCTURAL AND CHEMICAL PROPERTIES OF THE PLASMALEMMA OF *AMOEBA PROTEUS*[1, 2]

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The plasmalemma of *Amoeba proteus* is the limiting surface coat, which in the living animal is in close contact with the underlying hyaline layer of cytoplasm. According to the observations of Mast and others (14, 15) the plasmalemma plays an important role in the attachment and movement of the animal on solid surfaces as well as in the regulation of permeability by the amoeba. The aim of this paper is to study the structural and the chemical properties of the plasmalemma in order to arrive at a better understanding of the activities of this surface coat.

TECHNIQUE

Optical methods. The plasmalemma of living normal or centrifuged amoebae was examined with the following methods: 1) Phasecontrast microscope of the Zeiss Winckel model. 2) Darkfield microscopy by means of the Leitz parabolic condenser, provided with an azimuth diaphragm according to Heringa's method (point-lamp illumination) and with Lens-immersion with iris diaphragm. 3) Polarizedlight microscopy with a nicol Leitz microscope. 4) Retardation values were measured with the elliptical compensator according to Brace.

The amoebae were centrifuged according to the methods published by Mast, Andresen (1, 13) and others.

Fixation. These methods were also applied to the plasmalemma of fixed amoebae which were treated with the different fixing fluids: 1) Osmic solutions (osmic acid 2 per cent, Bensley's solution, Maximow's solution, Flemming's solution). 2. Formalin solutions of diverse pH. 3) Dehydrating liposolvents such as alcohol-acetone mixtures. 4) Solutions of salts of heavy metals such as Zenker's solution. 5) Solutions with sublimate. 6) Picric acid solutions such as Bouin's and Rabl's mixtures.

Optic observations were made on specimens of plasmalemma fragments obtained from fixed amoebae by means of micro-operations and by the use of fine pointed glass needles.

[1] Mit Unterstützung der eidg. Kommission zur Förderung der wissenschaftlichen Forschung aus Arbeitsbeschaffungsmitteln des Bundes.

[2] We are greatly indebted to Dr. H. E. Lehman (Chapel Hill, North Carolina, USA, at present Zoological Institute, Berne, Switzerland) for the revision of the manuscript.

Electron optical preparations. We have applied the same fragmentation methods (originally proposed by Lehmann and Biss (11)), to the fixed amoebae. One drop of the aqeous suspension of fragments was brought to the film mounted on the grid and there desiccated as fast as possible.

The electron microscope at our disposal was a Trüb-Täuber instrument used already in our preceding work.

RESULTS OF STRUCTURAL INVESTIGATIONS

Phase contrast microscopy. In regions, where the hyaloplasm is in a sol phase, the plasmalemma forms a sharp black line. It appears thickened, less clear and adherent to the underlying layer in zones where the hyaloplasm is in a gel-like state. Centrifugation does not modify the optical properties. However, in centrifuged amoebae, the plasmalemma of centrifugal portions appears to be very thin. In fact, it is there that rupture always occurs, when the amoebae are squeezed.

The optical properties of the plasmalemma are not essentially modified by osmic fixing fluids and isotonic formalin. Strongly precipitating or alcoholic fixing fluids induce the plasmalemma to become wrinkled, but without much change of the optical properties (Fig. 1 a). The pieces of plasmalemma, obtained by fragmentation of fixed amoebae appeared uniformly transparent. Their margins strongly refracted light. Probably due to the fixation procedure variable quantities of granula and hyaloplasm are found sticking to the surface of the plasmalemma fragments.

Darkfield microscopy. The plasmalemma of living amoebae strongly diffracts light and produces a highly luminous picture. The diffraction is not completely homogeneous but shows an interference phenomenon with different spectrum colours. These properties of the plasmalemma are not changed by centrifuging.

The plasmalemma of fixed amoebae shows a stronger diffraction than in living amoebae, especially along the margins of pieces, obtained by fragmentation of already fixed amoebae. The fragments by themselves are very transparent with weak diffractive spots. This picture is partly due to the plasmalemma itself and partly to hyaloplasm material, adherent to the internal surface of the fragment. (Fig. 1 b). There is no modification of the diffraction pattern by varying the light angle with the azimuth diaphragm.

Polarized light microscopy. W. J. Schmidt (19, 20, 21) who first studied the amoeba with polarized light, demonstrated in the plasmalemma positive birefringence in relation to the tangent. With crossed nicols, the plasmalemma appears to be luminous along the margin, while the central part is optically

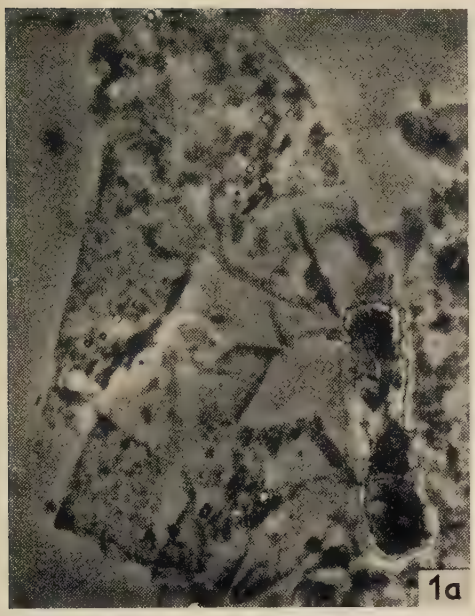
isotropic (the optical axis of the plasmalemma). In addition to this, we have observed at points where the amoeba appears most flattened, the plasmalemma as a very thin line and slightly luminous along the margins of the smaller pseudopodia. The plasmalemma of centrifuged amoebae appears brighter in accordance with the characteristic rounded shape of the amoeba induced by centrifugation.

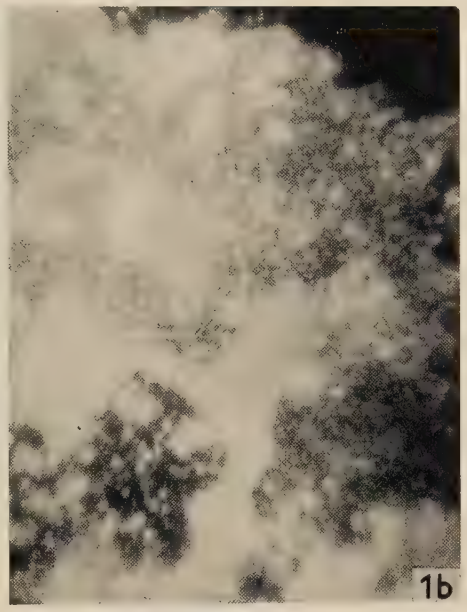
Brace's elliptical compensator permitted us to measure the retardation of living amoebae, which is about 15 millimicrons. Amoebae treated with osmic acid or some lipoid solvents give retardation values which were nearly the same as in the living animal, whereas amoebae, treated with strongly precipitating liquids (fixing fluids with heavy metals or Bouin's mixture) produced a marked increase in the above mentioned retardation values.

The double refraction of the plasmalemma is strongly influenced by imbibition of liquids having different refractive indices. Fig. 7 shows the change of double refraction in the same amoeba. This individual was first fixed in osmic acid and afterwards imbibed some miscible liquids of different refractive indices, beginning with distilled water up to methylene iodide, which has a refractive index of 1.72. (See Fig. 7, p. 231.)

When plotted as a curve, the experimental data showed that a refractive value of 1.57 and 1.59 was correlated with the lowest, indeed scarcely appreciable, retardation value of the plasmalemma. With crossed nicols at these refractive values, no luminous picture is seen nor are there modifications noticeable after varying the compensator position. The same observations were made with amoebae fixed in Bouin's solution, Zenker's acetic, formalin, alcohol, acetone.

Our data show conclusively, that the double refraction of the plasmalemma is not intrinsic birefringence, as it was heretofore believed, but for the most part is a "textural birefringence" a term proposed by A. Frey-Wyssling (6). An actual component of intrinsic birefringence could not be demonstrated by our methods. Mitchison (18) admits the existence of positive textural birefringence and negative intrinsic birefringence in amoeba. Unfortunately, Mitchison's analysis is based only on an incomplete study of the double refraction. Amoebae were first fixed in formalin and afterwards treated by mercury iodide with a refractive index of 1.56. This salt is, however, not soluble in water and the author does not give exact data on the manner in which complete imbibition of mercury iodide by the amoeba was accomplished. It is therefore not clear, whether the phenomenon described by Mitchison is caused by real intrinsic negative birefringence or rather by an incomplete imbibition of the plasmalemma.





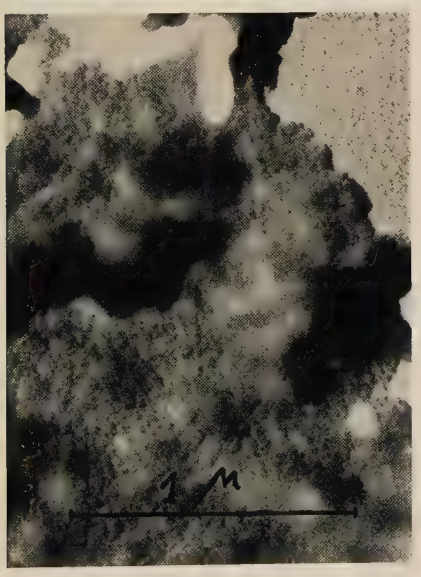


Fig. 1. Fragments of plasmalemma after fixation. A. Fixed with Zenker's fluid, photographed with phase contrast. 940 ×. B. Fixed with Bouin's fluid, photographed with dark field. The luminous points are not only due to diffraction of plasmalemma, but also to adhering residues of hyaloplasm. 1 200 ×.

Fig. 2. Electron micrographs of the external surface of plasmalemma after fixation with Zenker's fluid containing acetic acid (pH = 2,5). The globular appearance of surface is very clear. Shadowed with gold-manganine: A: Transparent picture. B: The same as shadow picture (after inversion).

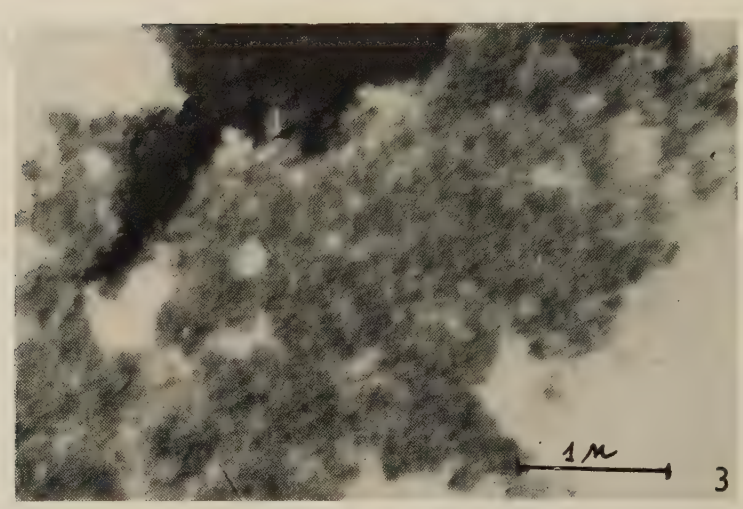


Fig. 3. Electron micrograph of the external surface of plasmalemma after fixation with Bensley's fluid. The globular appearance is less clear, rather mucous. Shadowed with gold-manganine.

Electron microscopical data. Dried fragments of plasmalemma are permeable to electrons. They show irregular, rather transparent fields, which are bounded by denser reticular structures. Similar pictures are obtained even after fixation with very different fixing fluids. Only the thickness of the fragments is variable following different types of fixation, the differences depending upon the effect of the fixative upon the adhering hyaloplasm.

The gold shadowed preparations show clearly two types of surfaces: A smooth regular surface, which is finely granulated and which represents the external one, and a rather coarse irregular surface, which may be regarded safely as the internal one.

The appearance of the external surface is in most preparations very similar, even after treatment with different types of fixing fluids. It seems to consist of densely packed minute globules forming a sintered globular film. The maximum number of globular protrusion is observed after precipitation by acid fixing fluids (Fig. 2). Other fixing fluids produce a rather cloudy picture without entirely suppressing the globular pattern (see Fig. 3). It is also visible after treatment with lipoid solvent agents.

The trabecular formations of the internal surface vary strongly in their structure according to the different procedures applied. Coagulating and precipitating fixing fluids produce denser and coarser, whereas formalin and osmic acid give less dense formations (see Fig. 4).

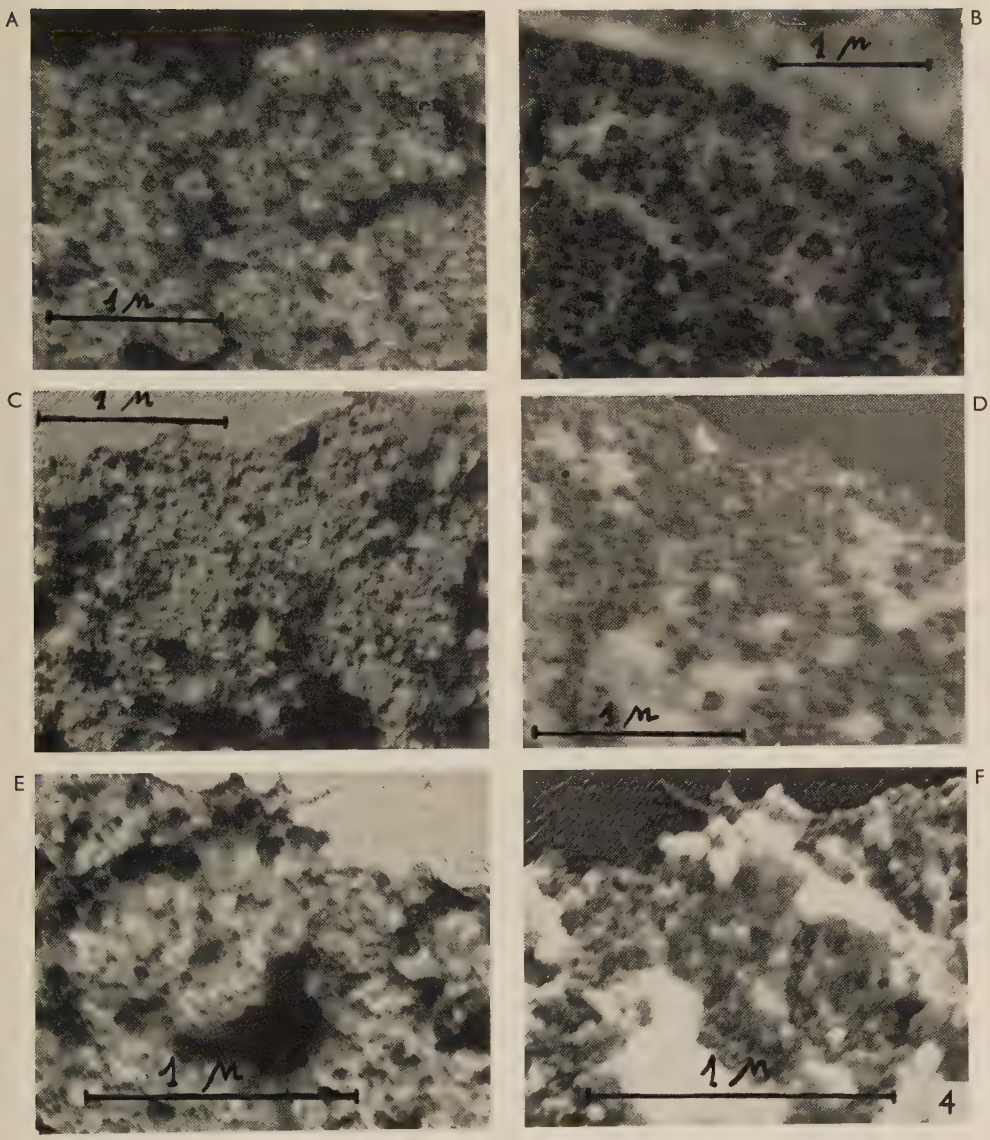
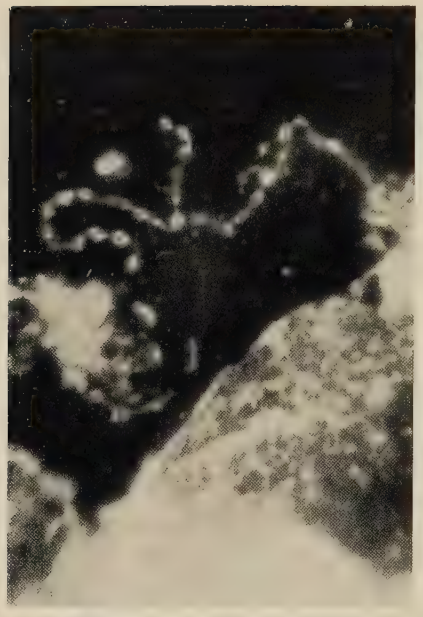
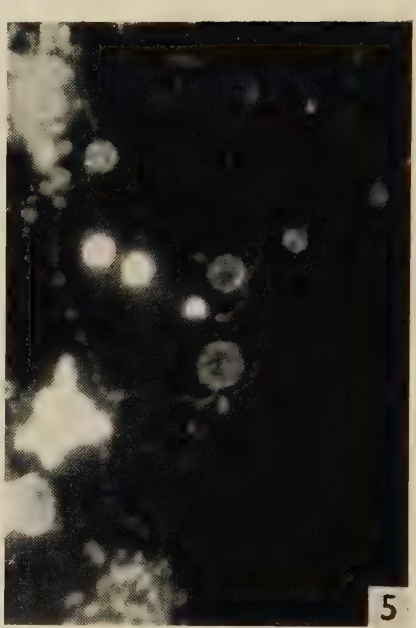


Fig. 4. Electron micrographs of the internal surface of plasmalemma after different fixations: the pictures at the same level show the same region of a preparation, at right the shadowed (inversion) picture, at left the transparence picture. A and B: fixation acetone-alcohol mixture. Coagulated reticulum of hyaloplasm very clear; there appears also the mucous external surface. C and D: fixation in formalin, afterwards treated with osmic acid. The reticulum appears much thinner than in A. E and F: fixation in acidified Zenker (pH = 2,5). The reticulum of the hyaloplasm shows coarse globular structures.

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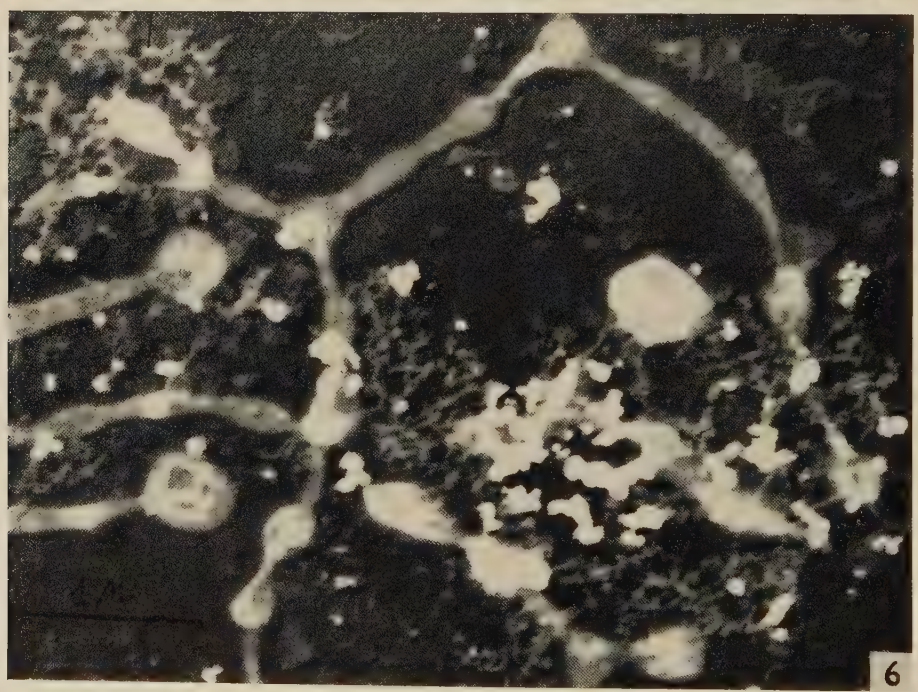
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In general the trabecular formations of the internal surface are identical to those of isolated hyaloplasm, and may therefore be regarded as hyaloplasm precipitations, adhering to the plasmalemma. They do not seem to be fully equivalent to the living hyaloplasm structures.

The fragments of plasmalemma never displayed deformed or unravelled filamentous structures near the artificially broken margins. Those margins have quite neat edges which are slightly dentate. They indicate also a globular pattern of the plasmalemma, whereas no signs of a real texture of filaments are to be found. (See Fig. 2 A, B.)

From shadowed preparations the thickness of the plasmalemma can be calculated. It is nearly 500 Å.

The plasmalemma tested by histochemical methods.

Autolysis. The plasmalemma immediately undergoes autolysis when an amoeba is compressed between two glass surfaces until the plasmasol flows out. This lysis induced by pressure develops in a few minutes and occurs in Ringer's, other saline culture, and sugar solutions. During autolysis, dark field observations show long filamentous formations containing rows of granules or densely-walled vesicles containing liquid. (Fig. 5.) The structures disappear after a short time if a slow flow of liquid is produced between the two glass surfaces. The electron micrographs (Fig. 6.) confirm the dark field observations: Threadlike formations with rows of vesicles, which resemble somewhat the myelin figures (Nageotte). However, these structures do not seem to be soluble in lipoid solvents nor have they negative intrinsic birefringence. Consequently there is as yet no indication of a lipoid nature of the plasmalemma.

Action of raised temperature. The amoebae are very sensitive to raised temperatures. At 37° C the amoebae become rounded, between 37° C and 42° C they appear paralysed and do not form any more pseudopodia. If the period of increased temperature does not last too long, the amoebae may return to a normal state within about half an hour. Between 48° C and 50° C the amoebae in most cases show autolysis. The lysis induced by raised temperatures brings about the same filamentous structures containing rows of globules as does autolysis after squeezing. A rapid rise in temperature

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Fig. 5. Photograph of myeline-like figures of autolyzing plasmalemma. Fixation by osmic acid 5 minutes after beginning of lysis. Dark field. 1 200 ×.
Fig. 6. Electron micrographs of myeline-like figures of autolyzing plasmalemma. Fixed with osmic acid 5 minutes after beginning of lysis.

up to 54–56° C quickly destroys the plasmalemma; except a few filiform formations with globules, which appear to be irreversibly coagulated.

The plasmalemma, if previously treated with fixing fluids, becomes more heat resistant. Specimens first fixed with alcohol or formaline and then exposed to water at a temperature of 70° C for some minutes will develop lysis. Osmic acid, Bouin's and Zenker's solutions (acidified or not) increase the heat resistance to such a point, that only a constant temperature of 85° C acting for several minutes will induce lysis.

Action of enzymes. It has not been possible to destroy *the plasmalemma of the living amoeba.* It resists pancreatin solutions of pH 7.5 and testicular hyaluronidase. Pepsin solutions damage the amoeba more by the low pH than by enzyme action, as was shown by some control experiments. Also experiments with mesomucinase made up in different solutions showed no action of the enzyme on the living amoeba. With a solution containing the enzyme dissolved in phosphate buffer of pH 6 or Ringer's, disintegration of the plasmalemma was obtained within 10 hours. This was accompanied by scattering of the body contents, nevertheless the vacuoles, granules, nucleus, etc. appeared to be essentially unaltered. However, the same effect is produced by saline solutions, without enzymes. Mesomucinase dissolved in amoeba culture medium, previously heated to 70° C, produced changes of form of the amoeba but did not attack the plasmalemma.

The plasmalemma of fixed amoebae possesses different degrees of resistance to various enzyme actions. Amoebae fixed in alcohol or acetone are digested in alkaline pancreatine solutions at a temperature of 30° C in about 24 hours, whereas pepsin (pH 2.5) does not act, even during 1–2 days. Amoebae fixed in Bouin's or Zenker's solution are very slowly attacked by pancreatine. If the amoebae are pin-pricked so as to permit a rapid penetration of this enzyme solution into the interior of the amoeba, a rapid lysis of the amoeba takes place. In this case however, the plasmalemma resists disintegration longer than the rest of the amoeba.

The hyaluronidase (Kinetin) shows a rather specific effect since within about 14 hours it attacks and disintegrates only the plasmalemma of amoebae fixed in alcohol or acetone.

Metachromatic and histochemical reactions. The plasmalemma is metachromatically colored by some basic dyes. It becomes red-orange in color in amoebae previously fixed in alcohol, acetone or Bouin's fluid when stained with a diluted toluidine blue solution ($^1/1000$ up to $^1/5000$). Spek and Gillisen (24) have already found the strong metachromatic reaction of the plasmalemma after staining with some basic dyes. Strong solutions are

absorbed to such an extent as to greatly modify the physical properties of the plasmalemma which finally may be shed.

The application of Hotchkiss' method results in pink-color of the plasmalemma. The oxidizing action of periodic acid with subsequent action of Schiff's reagent demonstrates the presence of carbohydrates in the plasmalemma.

The metachromatic reaction of the plasmalemma as well as the positive reaction of Hotchkiss indicate strongly the presence of mucopolysaccharides in the plasmalemma.

DISCUSSION

The chemical nature of the plasmalemma. All our observations suggest the conclusion that the plasmalemma of the amoeba is a coat rich in mucoproteids.

The direct proofs are the following ones: 1) strong metachromatic coloration by some basic dyes; 2) presence of carbohydrates shown by a positive Hotchkiss reaction; 3) the specific action of hyaluronidase upon the plasmalemma of fixed amoebae; 4) the solubility in water at raised temperatures: 5) the rapid post-mortal lysis.

It might be recalled that mucopolysaccharides are often united to their enzymes (the hyaluronic acid and hyaluronidase system). It seems very likely, that in the moment when the plasmalemma and cytoplasm of the amoeba are greatly damaged, the equilibrium of the hyaluronidase system in its synthesizing and disintegrating activities becomes disturbed in such a way that disintegration dominates and lysis occurs. The meaning of the myelin-like structures which are formed during lysis of the plasmalemma cannot be explained at this time. It is rather probable, that those structures do not contain lipoids (see p. 227).

Some other data also support our conclusions: Andresen (1) showed by his staining experiments, that the plasmalemma contains very few lipoids if any. Lipoid solvents do not modify the structure of the plasmalemma. On the other hand the presence of fibrillar proteins could not be demonstrated, either by polarizedlight microscopy or by enzyme actions.

It is of course not possible to decide whether mucopolysaccharides are the only components of the plasmalemma or whether they are combined with proteins. But at least the data obtained till now by several other authors and by ourselves, seem to demonstrate conclusively that the principal constituents of the plasmalemma are mucopolysaccharides.

The submicroscopical structure of the plasmalemma. Work done in the past

with polarized light seemed to indicate that the plasmalemma is a coat made up of a fibrillar sheet-like protein layer. However, our direct observations with the electron microscope are not in favor of this concept. Instead of a fibrillar film, we have found the external surface of plasmalemma to be clearly a film of densely packed or sintered globular particles, whereas the internal surface shows a less clear pattern of globular particles.

It is of course a question, as to how far the electron optical pictures are equivalent to the living state of the plasmalemma. In the case of the plasmalemma, the globular pattern could not be significantly altered even by very different fixing fluids. We believe this to be a good evidence for the real existence of the globular pattern of the plasmalemma.

There exist, however, some differences in the reactions of the plasmalemma to fixing fluids containing acetic acid, and those which are not strongly acid. The very clear pattern, which is produced by acid fluids may result from a partial solution of an outer layer of a mucous material, whereas this material is preserved by less acid fluids. If this were true, it might account for the more cloudy appearance of plasmalemma, as described in the preceding section.

Another important question is, whether or not the globule-containing film is the only constituent of the plasmalemma. It is our opinion that the electron microscope by itself cannot answer this question decisively, since there may exist very thin protein layers between the external surface coat and the hyaloplasm which cannot be resolved by the electron microscope. (See Fig. 8.)

From the polarized light data (see Fig. 7) we have seen that the plasmalemma is anisotropic and that its textural anisotropy cannot be attributed to the intrinsic structure of the molecular constituents of the plasmalemma. It is very likely that the space arrangement of the structural constituents represents a "mixed body" (Mischkörper) of complex structure. If we accept that the electron optical picture represents in a rather equivalent manner the globular pattern, we are able to explain the textural birefringence. In fact, a layer composed of globular bodies with a diameter less than that of the light wave length may produce such birefringence. Furthermore, the coagulated structures of hyaloplasm, adhering to the internal surface of the plasmalemma may produce an arrangement of a "mixed body" (Mischkörper) in the sense of Wiener. The fact that the different fixing fluids induce a varied birefringence intensity may be explained then by the varied density of the adhering trabecular layer.

As a whole, our data seem to be well in favor of the globular pattern

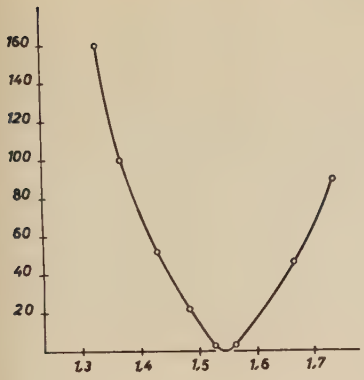

Fig. 7.

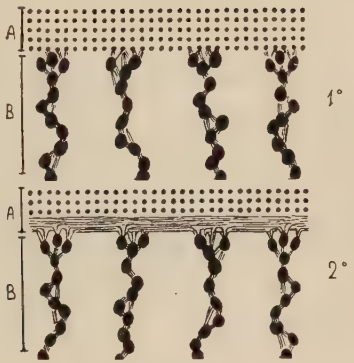

Fig. 8.

Fig. 7. Curve of retardation values of the plasmalemma, measured with the compensator of Brace in the same amoeba after imbibition with successive liquids of different refraction index.[1] On the abscisse are marked the values of refraction, on the ordinate the retardation values. The curve is a typical case of a textural birefringence curve.

Fig. 8. Diagrams demonstrating two possible sub-microscopic architectures of plasmalemma. The dimensions of the layers are not calculated but roughly estimated. 1) Diagram of a purely globular architecture of the gelated plasmalemma (A; to its internal surface the hyaloplasm reticulum is attached as it appears from fixed preparations (B). 2) Diagram of the sub-microscopic structure formed by a globular layer and by a protein-filament layer (A) which continues with the hyaloplasm reticulum as it appears from fixed preparations (B).

concept of the plasmalemma. But we are aware that several points in our considerations are open to criticism. It might be said that we did not take into account sufficiently the minimum intrinsic birefringence. If there is any, however, it might be explained by the presence of an internal protein layer (Fig. 8). Another controversial point is the question of whether or not fixing fluids influence birefringence essentially. For obvious technical reasons one could not avoid fixing the amoebae prior to determining textural birefringence. In the future it has to be decided, how far the fixing methods fundamentally change the structure of the plasmalemma. It has to be recalled however, that very different fixing fluids preserve the structure of the plasmalemma in a similar manner.

Adhesiveness and chemical structure of plasmalemma. As Mast (13) has shown, the adhesiveness of the plasmalemma of the amoeba is influenced by two groups of factors. External factors such as different salts, lactose, glycerine and urea facilitate attachment of the animals, whereas amoebae attach very slowly in chemically pure water. On the other hand attachment

[1] Refractive indices of liquids used: Distilled water = 1,33; Acetone = 1,35; Alcohol abs. = 1,38; Chloroform = 1,44. Xylol = 1,49; Benzol = 1,50; Cedar oil = 1,54. Chloroform + alphamonobromonaphthalin = 1,57; alphamonobromonaphthalin = 1,65; methylene iodide = 1,72.

depends on the physiological condition of the amoebae. In some specimens in a given solution the plasmalemma is very much more adhesive than others in the same solution. The favorable effect common to salts and urea on attachment must be associated with some common property, probably the electric charge carried by the ions which may change properties of the surface coat and at the same time influence internal processes. It is an interesting question how the changes of adhesiveness are correlated with changes in the mucopolysaccharides and the enzyme system of the plasmacoat of the amoeba. Our new findings might be of use for a further analysis of this problem.

SUMMARY

1. The structural and chemical properties of the plasmalemma of *Amoeba proteus* were investigated with optical, electron optical and histochemical methods. Normal and centrifuged amoebae were used as well as specimens fixed with different fixing fluids.

2. The double refraction of plasmalemma is positive in relation to the tangent. Imbibition with liquids of different refractive index brings about considerable changes in double refraction. It is concluded from the curve of refraction values that the plasmalemma shows birefringence which is for the most part "textural" (Formdoppelbrechung).

3. In electron optical preparations the external surface of the plasmalemma appears to persist even after treatment with very different fixing fluids, as a sintered globular film, formed by densely packed minute globular bodies. The thickness of the film is about 500 Å.

4. The following histochemical tests suggest the conclusion that the plasmalemma contains a great amount of mucopolysaccharides, probably coupled to proteins: strong metachromatic colorability by some basic dyes; presence of carbohydrates as shown by a positive Hotchkiss reaction; the specific action of hyaluronidase upon the plasmalemma of fixed amoebae; the solubility in water at raised temperatures; the rapid postmortal lysis.

5. The experimental data obtained might be explained by the following assumption. The plasmalemma is a mucous surface coat composed predominantly of globular bodies (probably mucoproteids). The diameter of the single body is considerably less than the wave length of light; therefore the whole complex of these minute bodies gives textural birefringence. The adhering layers of hyaloplasm may act, together with the surface coat as a "mixed body" (Mischkörper) and influence birefringence according to the various precipitating action of fixing fluids.

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A CARTESIAN DIVER BALANCE-CAPILLARY RESPIROMETER FOR METABOLIC STUDIES OF TISSUE CULTURES[1]

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The quantitative physiological study of tissue cultures is entirely dependent on the development of adequate methods. These methods must be sufficiently precise for application to the small tissue samples used and at the same time must meet the rigid requirements for optimum growth of cultures. In addition to the requirement of relatively high precision, adequate methods must ensure a minimum of manipulation of the tissue, absence of injury by drying, absolute sterility, and optimum conditions of oxygenation and nutrition.

Several attempts have been made to measure the respiratory metabolism of tissue cultures (3, 4, 10, 11, 15, 16, 18, 19, 20, 21, 22, 23, 24). In general these methods have failed to meet one or more of the above requirements, the most common shortcoming being an inadequate measure of the quantity of tissue used. Furthermore, in no case has close correlation between morphological changes and the metabolism associated with those changes been possible.

This paper describes the design, construction, and rigorous testing of an apparatus which allows continuous measurement of respiratory rate per weight of tissue and periodic microscopic observation of the morphology of tissue cultures throughout their growth period. The apparatus is a combination of two previously described devices. A modification of the Zeuthen Cartesian diver microbalance (25) is used for weighing tissue explants, and a modification of the Gregg capillary microrespirometer (6) is used for measurement of gas exchange of the tissue.

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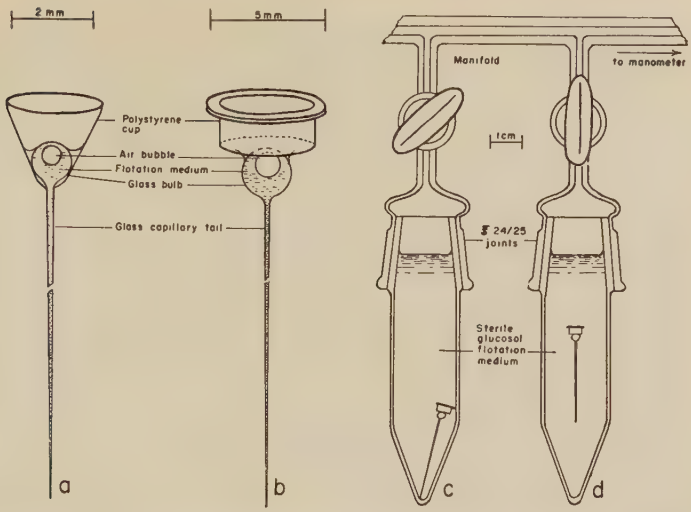

Fig. 1. Cartesian diver microbalance for weighing tissue explants. a. Original Zeuthen diver balance. b. Diver balance modified for use as culture and respirometer vessel. c. and d. Diver balances in flotation vessels attached to manifold. The diver balance in "d." is in the equilibrium position for measurement of *e.p.*

DESCRIPTION OF APPARATUS

The balance. The theory of the use of the Cartesian diver as a respirometer is discussed in detail by Linderstrøm-Lang (13). The use of the diver as a weighing device was introduced by Zeuthen (25). Briefly, the principle of the diver balance depends on the relationship between the "floatability" of the diver and the pressure exerted on the flotation vessel (see Fig. 1). The pressure necessary to hold the diver in a stationary submerged position as in Fig. 1-d (equilibrium pressure) is an index of the weight supported by the diver. By observing the equilibrium pressure (*e.p.*) first for the unloaded diver, then for the diver carrying a known standard weight, and finally for the diver carrying an object of unknown weight, the reduced weight of the unknown object — in this case a tissue explant — can be determined.

The equation used by Zeuthen (25) to relate equilibrium pressure measurements to reduced weights may be simplified as follows:
Zeuthen's equation I) is

$$\text{I)} \qquad RW_x = RW_{st}\left(\frac{1-\left(\frac{B}{B-P_x}\right)}{1-\left(\frac{B}{B-P_{st}}\right)}\right)$$

where RW_x = reduced weight of the unknown object, RW_{st} = reduced weight of the standard, B = atmospheric pressure in mm of manometer fluid = = 10.000, $P_x = e.p.$ for the diver loaded with the unknown object minus $e.p.$ for the unloaded diver $= e.p._x - e.p._0$, and $P_{st} = e.p._{st} - e.p._0$.

Equation I) reduces to:

$$RW_x = RW_{st}\left(\frac{\frac{B - P_x - B}{B - P_x}}{\frac{B - P_{st} - B}{B - P_{st}}}\right) = RW_{st}\left(\frac{-P_x}{B - P_x}\right)\left(\frac{B - P_{st}}{-P_{st}}\right)$$

II) $$RW_x = RW_{st}\frac{P_x}{P_{st}}\left(\frac{B - P_{st}}{B - P_x}\right).$$

Since B is very large (equals 10.000) with respect to P_x and P_{st}, the last term of equation II) is approximately equal to unity. If P_x and P_{st} do not differ by more than 100 mm., this approximation is within 1 per cent and equation II) can be reduced to the much simpler form:

III) $$RW_x = RW_{st}\frac{P_x}{P_{st}}.$$

The size of the standard weights was chosen so that their reduced weights do not differ greatly from the reduced weights of the tissue explants used; in no case was the difference between P_x and P_{st} greater than about 50 mm. Hence the approximate form, equation III) is sufficiently accurate and was used in these experiments.

The Zeuthen diver balance, shown in Fig. 1-a, has been modified as indicated in Fig. 1-b. The sensitivity has been decreased to a value appropriate to the size of tissue explants used by tripling the size of the diver. The diver cup is machined from polystyrene rod to the form shown, having a flat bottom to serve as a surface substratum for the growing culture and a flanged lip to provide a means of attachment to the capillary respirometer (see below). This cup is attached to the Pyrex bulb by cementing with dissolved plastic. The details of blowing the bulbs and filling the bulbs with flotation medium are exactly as described by Zeuthen (25).

Standard weights. Small cylinders (about 1 mm long by 1 mm diameter) were machined from a polystyrene rod, the density of which had been accurately determined by measurement of its volume and weight. The small cylinders were polished on ground glass, then were weighed on a sensitive microbalance to within ± 0.003 mg. The reduced weights of these

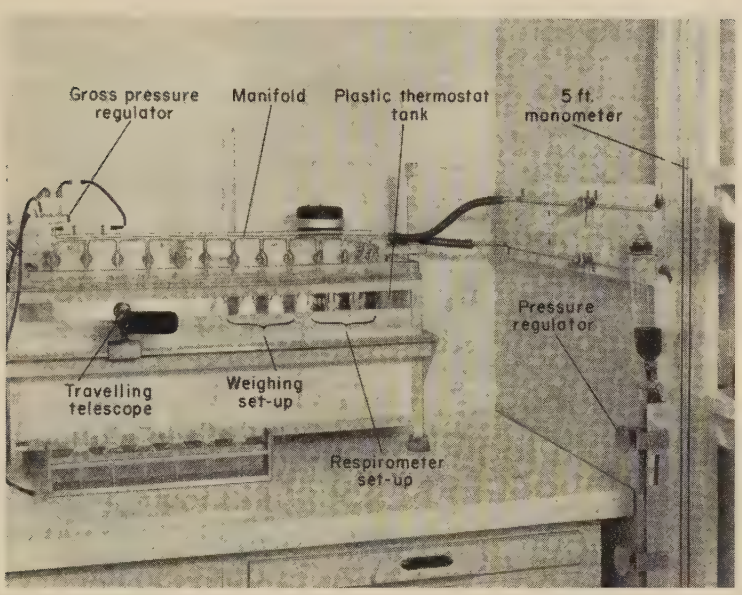

Fig. 2. Experimental setup for use of the Cartesian diver microbalances and capillary microrespirometers in tissue culture studies.

standards were calculated, using Linderstrøm-Lang and Holter's (14) formula relating reduced weight to absolute weight, density of the flotation medium, and density of the weight: $RW = W_A\,(1 - d_m/d_{wt})$. The density of the polystyrene, d_{wt}, is 1.0484 and the density of the medium, d_m, is 0.9985, both at 37.5°. The absolute weights of the standards are slightly over 1 mg, and their reduced weights are about 55 μg.

Flotation vessels and medium. The flotation vessels were made by forming conical tips on the shanks of T 24/25 inside joints. The vessels, containing the diver balances, and their attachment to the manifold are indicated in Figs. 1-c and 1-d.

Glucosol[1] was used as the flotation medium for weighing the tissue explants because it contains no bicarbonate and can, therefore, be sterilized by autoclaving. The density of the glucosol was determined after autoclaving by pycnometer measurements at the temperature of the thermostat.

Experimental setup. The manometer, compensation vessel, manifold and thermostat (Fig. 2) are in principle the same as described by Holter (7)

[1] The composition of glucosol is: 8.0 g NaCl, 0.20 g KCl, 0.20 g anhydrous $CaCl_2$, 0.10 g $MgCl_2 \cdot 6H_2O$, 0,05 g $NaH_2PO \cdot H_2O$, and 1.00 g glucose in 1 liter of glass distilled water (1).

and by Zeuthen (25). However, several modifications were introduced in order to use the apparatus at 37.5° as required for mammalian tissue cultures. The large difference between the temperature of the thermostat and that of the surrounding air required a more compact design of the manifold and better insulation of both the manifold and the thermostat tank.

The thermostat consists of a small tank within a large tank. Both are constructed of transparent plastic, the inner one 1/8 inch thick and the outer one 3/8 inch thick. The walls of the outer tank are covered with asbestos except for the viewing window, and the top is covered with a tight fitting 3/8 inch thick lid. The outer tank is maintained by usual methods at 37.50° $\pm$0.01° C., while the inner tank, which has no heating element, depends entirely on the surrounding water of the larger tank for constancy of temperature. Immediately after the inner tank is opened and the flotation vessels are introduced, the water is circulated from the outer tank through the inner tank until temperature equilibrium is attained. During operation the water in the inner tank is not stirred. This arrangement of the double tank was found necessary in order to avoid undue fluctuation of temperature during the weighing procedure.

The twelve position manifold is enclosed in a 1/4 inch thick plastic case which also serves as a tight lid for the inner thermostat tank. This case gives mechanical support as well as insulation to the fragile glass manifold.

The whole apparatus is set up as shown in Fig. 2 in a sterile culture room maintained at 28° $\pm$ 2° C.

The respirometer. The principle of the Gregg (6) capillary microrespirometer (Fig. 3-a) has been used with the modifications shown in Fig. 3-b. Again, the size has been increased in order to decrease the precision. The device has been further changed by eliminating the depression in the capillary tube and using instead the cup of the diver balance as a respirometer chamber. The cup is attached to the flange of the capillary by sterile petrolatum-lanolin, and the upper end of the capillary is closed off by an index drop of colored fluid (Fig. 3-b). The position of this index drop is held constant by manipulation of the external pressure on the open end of the capillary (Fig. 3-d). Thus the device is a constant volume respirometer and has theoretical characteristics exactly comparable to the usual macro Warburg apparatus. The only difference is the much smaller volume and, therefore, much smaller flask constant.

The precision of the respirometer depends on both the total volume and the bore size of the capillary. The first determines the precision with which gas uptake can be read as pressure differences on the manometer. The

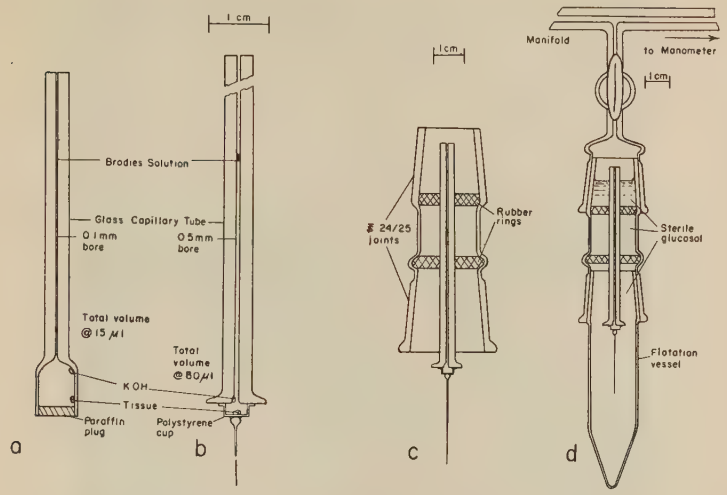

Fig. 3. Capillary microrespirometer for measuring the respiratory metabolism of tissue cultures. a. Original Gregg respirometer. b. Respirometer modified to use diver balance as respirometer vessel. c. Respirometer mounted in T connecting tube. d. Respirometer assembly (connecting tube and flotation vessel containing respirometer) attached to manifold.

second determines the precision with which the volume can be held constant. The capillaries were constructed from nominally 1/2 mm bore thick walled Pyrex tubing, selected for actual bore size of 0.3 to 0.35 mm, which has a bore cross sectional area of about 0.1 square mm. With this size capillary and an estimated error of setting the index drop (on the cross hair of the travelling telescope of Fig. 2) of about 0.1 mm, the volume error of setting the index drop at the index position is about 0.01 µl. The total volume of the respirometers was determined by weighing and calculating the volume of mercury contained. The volume is about 80 µl, giving a K_{O_2} of about 0.01 µl 0_2 per mm of manometer fluid pressure difference. Thus both limits of precision are of about the same order and allow a gas exchange of 1 µl to be measured within 1 per cent error.

Respirometer mounting. For convenience in use, the capillaries are mounted in T 24/25 connecting tubes as indicated in Fig. 3-c. The same thermostat, manifold and manometer are used for respiration measurements as for weighing. Hence, the dimensions of this tube are critical in that the 1 cm transparent cylindrical part of the tube must fall in the line of sight of the telescope when the tube is attached to the manifold and is in place in the thermostat, as in Figs. 3-d and 2.

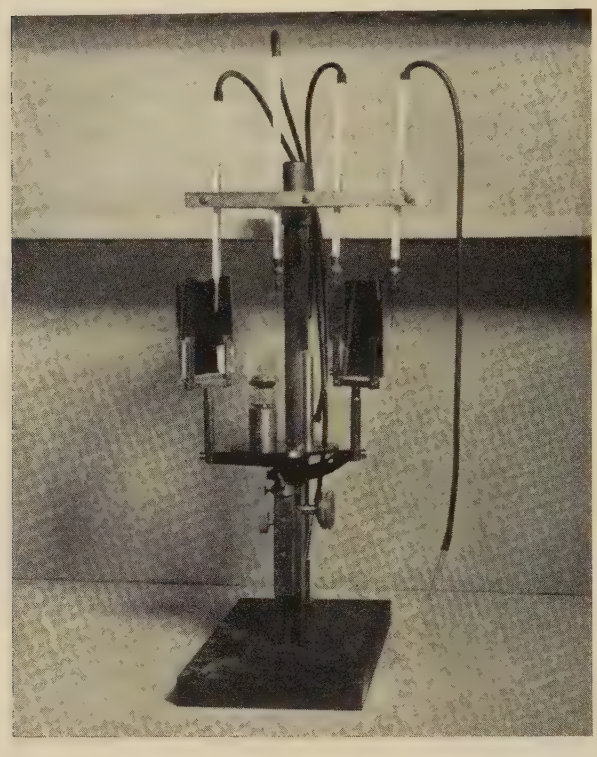

Fig. 4. Pipeting device.

Pipets and pipeting device. Pipets were made from thermometer capillaries, either drawn out to fine tips as described by Holter (7) or adapted to fit hypodermic needles. The needle tips are much less fragile and are used for all noncorrosive fluids. The pipets were calibrated by the iodate-thiosulfate titration method of Linderstrøm-Lang and Holter (14).

A pipeting device similar in principle to the one described by Holter (7) was constructed and is shown in Fig. 4. A revolving stage, carrying brass mounts to hold either a respirometer in its connecting tube or a diver microbalance, is mounted on a rack and pinion. Above this is mounted a sliding bar by means of which each of four pipets can be centered over the respirometer capillary or the diver cup.

PROCEDURE FOR USE OF THE APPARATUS

The rather exacting routine described by Zeuthen for use of the Cartesian diver microbalance has been further complicated by the high temperature

and absolute sterility requirements of tissue culture. However, it has been found that by following the routine presented below the measurements can be made quickly and without unreasonable burden on the operator.

Preliminary. 1. Sterilize divers and standard weights by ultraviolet irradiation (2) and store in sterile distilled water.

2. Prepare by the usual methods 12 tissue fragments for culture explants.

Weighing procedure. 3. Place the flotation vessels in an auxiliary thermostat ($38.0°\pm0.5°$). Partially fill each vessel with glucosol (preheated to approximately 38°). Introduce the 12 numbered divers to the correspondingly numbered flotation vessels and adjust the volume of the air bubble in each diver so that it just sinks (25). Attach the vessels to the manifold, lower the manifold over the inner termostat tank, and allow 10 minutes for temperature equilibration. Observe the equilibrium pressure for each of the unloaded divers as follows: (a) Open the manifold stopcock connecting the particular flotation vessel to the manometer (all other manifold stopcocks closed). (b) Using the pressure regulator (Fig. 2, lower right), adjust the pressure until the diver just hangs in suspension, as observed in the travelling telescope. The diver must neither rise nor sink for a period of 15 seconds, and two readings of the *e.p.* are always made. (c) Read the level of the two arms of the manometer. The difference is the equilibrium pressure.

Remove in order the 12 flotation vessels from the manifold and introduce the correspondingly numbered standard weights to the diver cups; then replace the vessels on the manifold. Replace the manifold over the thermostat, equilibrate, and again read the *e.p.* for each of the 12 divers now loaded with its standard weight.

Remove the flotation vessels from the manifold in order. Remove each standard weight from the diver and replace it by a tissue explant. Replace the vessels on the manifold, replace the manifold over the thermostat, equilibrate, and again read the *e.p.* for each of the 12 divers now loaded with the tissue explant.

Respiration procedure. 4. Raise the manifold, remove the flotation vessels and remove the divers from the vessels. Pipet the glucosol out of the diver cup and slip a 5 mm diameter No. 0 cover slip under the explant (the cover slip enables easy removal of the culture for examination and permanent fixation). Place the diver in the mount in the pipeting device and add 3.2 μl of chicken plasma and 3.2 μl. of embryonic extract to form the culture clot. Place the correspondingly numbered respirometer connecting tube in its mount on the pipeting device and introduce 1.2 μl of 15 per cent KOH into the recess beside the capillary opening. Fasten the diver cup to the

respirometer capillary by petrolatum-lanolin (Figs. 3-b and 3-c), then join the flotation vessel and connecting tube. Add glucosol to the assembly to the level shown in Fig. 3-d and place the whole assembly in the auxiliary thermostat at 38°. Repeat with each of the respirometers and allow all to reach temperature equilibrium.

5. Add the index drop[1] by tuberculin syringe, using a needle cut to length so that the drop is always placed at the desired index position. Transfer the respirometer assemblies to the manifold and introduce them to the inner thermostat tank. Allow 10 minutes for temperature equilibration, then follow oxygen uptake by reading the manometer excursion necessary to return the index drop to the index position in the capillary as observed in the travelling telescope. Each of the 12 respirometers is connected in turn to the manometer as in the weighing procedure. Readings are generally made every 30 minutes for a 3 hour period. Continuous observation for longer than about 8 hours is not feasible because unduly large negative pressure is required to hold the index drop at the reference point.

6. After the 3 hour observation period, the respirometer assemblies are removed and the index drops withdrawn with a syringe and long needle. The assemblies are then returned to the thermostat until another measurement of respiratory rate is desired.

7. The respiration rate can again be determined at any time during the culture period by repeating steps 5 and 6. In the experiments for which this apparatus was designed, the respiratory rate was measured every 12 hours for a 3 day culture period.

PROOF OF METHOD

The apparatus and technique described here deviate somewhat from the original procedures described by Zeuthen and Gregg. Furthermore, the application to tissue culture, although following as closely as possible the accepted standards for optimum growth of isolated tissues, represents a departure from conventional methods. It is necessary, therefore, to establish the validity and applicability of the method in each of its details and in the composite. Experiments were carried out which verify the weighing, respiration and culture procedures separately and in combination.

Weighing procedure. The weights of the polystyrene standards remained essentially constant over a nine month period of continuous use. The greatest change in weight was an increase of about 1.5 per cent.

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[1] Any colored fluid is adequate. Brodie's solution was used in early experiments. In later experiments the more stable Kreb's solution (9) was used for index drops in the respirometers as well as for manometer fluid.

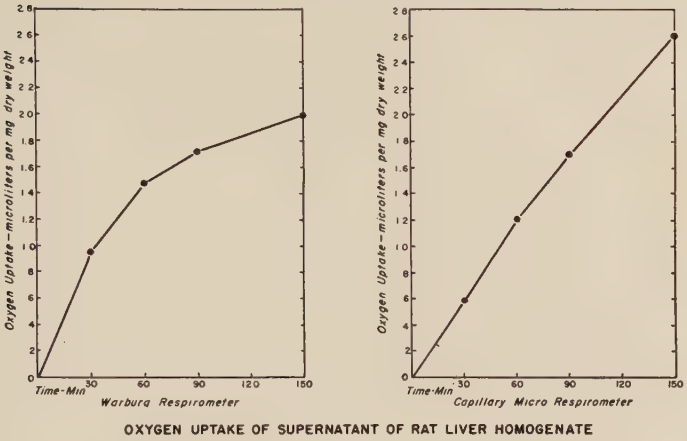

Fig. 5. Comparison of respiratory rate of supernatant from 20 per cent rat liver homogenate as measured by conventional macro methods and by the capillary microrespirometer. Two ml samples were used in 7 ml Warburg flasks; 5 μl samples in the microrespirometers. The curve for the macro method represents the average of 10 determinations and that of the macro method of 24 determinations.

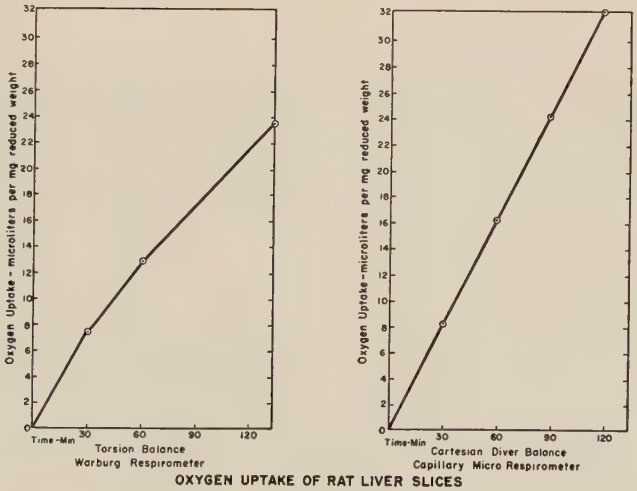

Fig. 6. Comparison of respiratory rate of rat liver slices as measured by conventional macro methods and by the Cartesian diver balance and capillary microrespirometer. Three slices weighing about 200 mg fresh weight were used in each Warburg flask: fragments weighing from 10 to 70 μg RW in the microrespirometers. Data for the macro method represents 12 determinations and for the micro method 24 determinations.

Constancy of equilibrium pressure was necessary for only a short time since the whole procedure for weighing 12 tissue explants takes less than one hour (steps 2 and 3 of the experimental procedure). Following the equilibrium pressure of unloaded

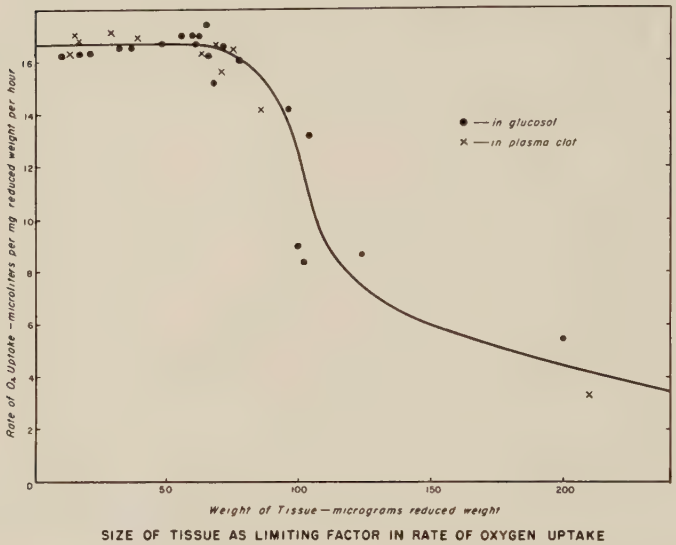

Fig. 7. Effect of tissue size and plasma clot on respiratory rate of rat liver slices.

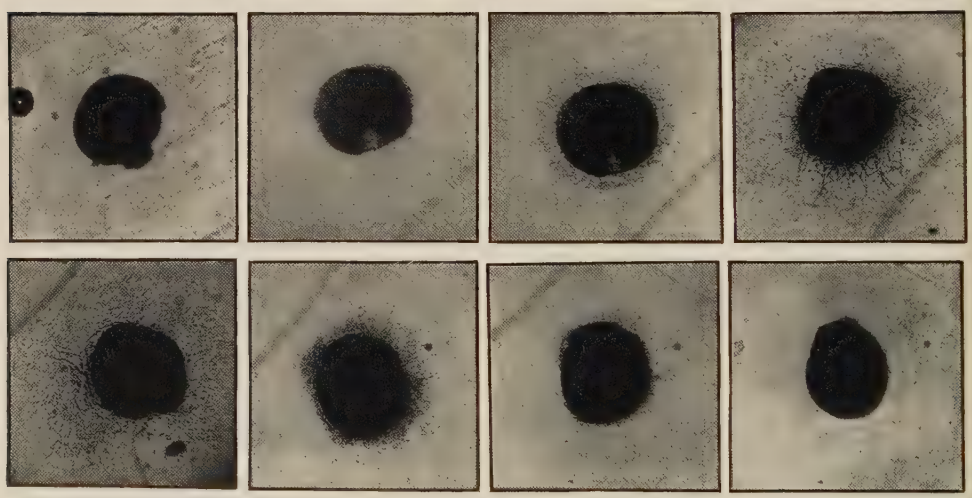

Fig. 8. Comparison of growth of 7 day embryonic chick heart cultures grown in hanging drop slides and in diver balance cups. Original magnification × 80.

divers at one hour intervals showed that no change could be detected during the first hour and a change of less than 5 mm was observed over six hours.

The accuracy of the weighing procedure was determined by comparing the measured reduced weights of combinations of polystyrene standards as obtained by the

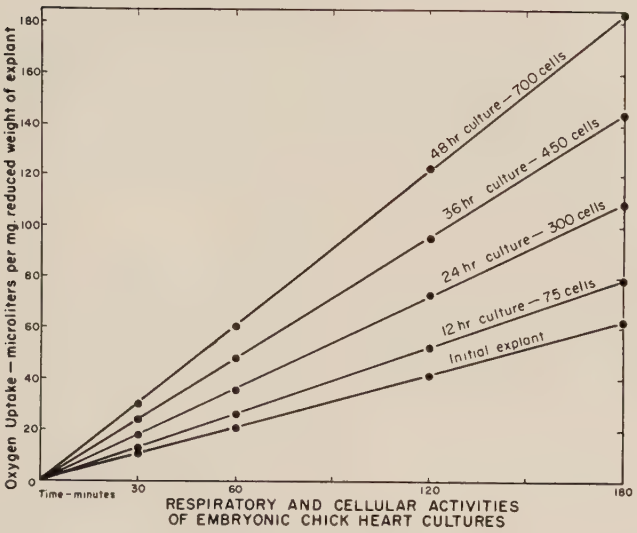

Fig. 9. Respiratory and cellular activities of 7 day embryonic chick heart cultures.

diver balance technique with their reduced weights calculated from the absolute weights. Fifteen of these standards were used in various combinations on each of the 12 divers. It was found that reduced weights in the range of 5 to 500 micrograms (approximately 0.1 to 8 mg air weight) could be determined reproducibly within $\pm$ ± 0.2 μg.

Respiration measurements. The rates of oxygen uptake of macro and micro samples of the supernatant of a 20 per cent rat liver homogenate were measured (Fig. 5). The only significant difference noted was the more rapid degeneration of the macro sample as indicated by falling rate of respiration. This greater stability of tissue in microrespirometry may be, at least in part, associated with the absence of vigorous shaking.

Combined weighing — respiration procedure. The respiratory activity per unit weight of macro and micro liver slices were measured simultaneously by the Warburg and capillary microrespirometer procedures respectively[1] (Fig. 6). The rates of the two methods were quite closely comparable. Again, as with the supernatant of the rat liver homogenate, the small tissue samples were more stable in the irrespiratory rate than were the large slices.

As shown in Fig. 7, there is a maximum size of tissue sample above which the rate

[1] In order to make this comparison, it is necessary to express the respiratory rates on the same weight basis. The observed fresh weights of the macro slices were converted to reduced weights by the formula: $RW_t = FW_t\,(1 - d_m/d_t)$ (13). The density of the liver as determined by weight and volume measurements (by means of a Lazarow manometer calibrator (12)) is 1.131. The density of the medium is 0.9985. Using these densities in the above equation, the relationship between reduced weight and fresh weight for rat liver is: $RW = 0.117\ FW$.

TABLE I

Comparison of respiratory rate determinations on embryonic chick heart cultures.

Source of data	Age of culture days	Q_{O_2} µl/mg d. wt./hr.
Warburg and Kubowitz (20)	3	22.9
	24	12.5
	over 1300	12.0
Meier (16)	1	19.7
Laser (10)	1	15.4
	5	12.7
	10	7.8
Lipmann (14)	1	27.5
	3	22.1
	over 30	13.6
From Fig. 8	0 (explant)	10.4
	1/2	13.2
	1	18.2

of oxygen uptake per unit weight of tissue becomes dependent on the size of tissue sample. It is apparent that below about 70 µg the respiration per microgram of tissue is independent of tissue weight, while above this size the oxygenation of the tissue is inadequate for useful measurement. In culture experiments, tissue explants of from 20 to 50 µg RW were used.

Fig. 7 also shows the results of experiments designed to test the possible effect of the plasma clot on the respiratory rate of tissue fragments. It is apparent from these measurements that the clot does not act as a gas barrier and has no influence at all on the rate of gas exchange.

Culturing procedure. Cellular activity of 7 day embryonic chick heart cultures grown in the diver cup and in the conventional hanging drop slide appear to be essentially the same (Fig. 8).

Combined weighing, culturing and respiration procedure. Fig. 9 shows the respiratory and cellular activity at 12 hour intervals of a series of 7 day embryonic chick heart cultures maintained for two days. During the culture period the respiratory rate increases as new cells are formed.

These Q_{O_2} values fall near the mean for reported respiratory rate determinations of chick heart in culture (Table I).

SUMMARY

The Zeuthen Cartesian diver microbalance and the Gregg capillary microrespirometer have been modified and combined into a single apparatus for use in biochemical studies of tissue cultures. Reduced weight as an

index of tissue quantity can be measured in the range of 5 to 500 μg to within 1 per cent error. Respiratory rates as low as 1.0 μl per hour can be measured with the same accuracy. A series of experiments was carried out which demonstrate the reliability and applicability of the weighing, respirometry and culturing techniques separately and in combination.

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BRIEF NOTES

EVIDENCE FOR THE CONVERSION OF DESOXYRIBONUCLEIC ACID (DNA) TO RIBONUCLEIC ACID (RNA) IN *EPISTYLIS ARTICULATA* FROM. (CILIATA: PERITRICHA)

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Turner (9) summing up his review on Fertilization in Protozoa remarked "The disintegration of the old macronucleus and the differentiation, number and distribution of new macronuclear anlagen have been noted, but no great significance has been attached to these processes. It now seems probable that more intensive investigation of the role of the macronucleus in conjugation will be extremely profitable" (p. 639). It is well known that in all ciliates where conjugation has been studied, the macronucleus 'disintegrates' and is absorbed in the cytoplasm while a new macronucleus arises from the division products of the synkaryon. Neither the significance of the degeneration of the old macronucleus nor the mode of development of the new, is known. It is also not clear if the two processes are causally related, though it has long been surmised, and some indirect evidence exists, they might be (1, 4, 6).

This note deals with the first of the two phenomena, the disintegration of the old macronucleus, in a peritrichous ciliate, *Epistylis articulata*. Among ciliates there does not appear to be any uniformity in the matter of this disintegration. In *Paramecium*, the macronucleus is drawn out into a long and complicated skein. This breaks up into fragments which are later absorbed in the cytoplasm. In examples like *Chilodonella* it remains as a single body which becomes gradually smaller and is finally lost to the view. The chemical change which attends on this breaking up and absorption is unknown. Staining reactions as well as ultra violet absorption technique have shown that the macronucleus has abundant amounts of DNA (3, 7, 8) and it is an interesting but unanswered question as to what happens to this DNA when the macronucleus breaks up and is absorbed in the cytoplasm. Speculations that it is in some manner transferred to the new macronucleus have been made (1, 4, 6), but no direct evidence of such a transfer has ever been found; nor the manner in which such a transfer could be effected. Recent analysis of certain ciliates has, however, shown that an indirect relationship might exist between the two, — the degeneration of the old macronucleus and the development of the new (8).

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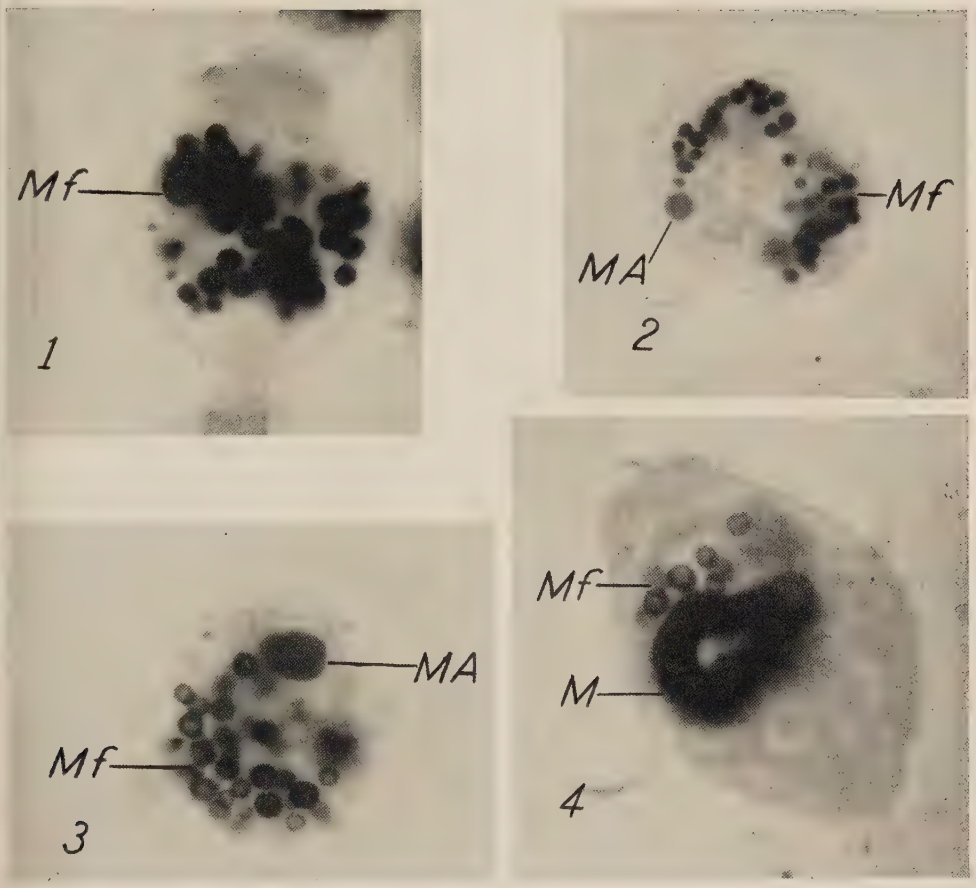

Fig. 1. Synconjugant of *Epistylis articulata*, early stage. The cytosome is filled with macronuclear fragments (Mf) which are solid and deeply stained. Feulgen. × 1100.
Fig. 2. Later stage synconjugant. A few fragments have developed spaces. A macronuclear anlage is seen (MA). Feulgen. × 1000.
Fig. 3. Later stage synconjugant. All fragments have developed Feulgen negative areas. Feulgen. × 1100.
Fig. 4. One of the products of the second reorganization fission. The macronucleus (M) has assumed its definitive form. The fragments are few and deeply vacuolated. Feulgen. × 1100.

A study of *Epistylis articulata* indicated that it was possible to explain the disintegration and absorption of the old macronucleus in terms of a fundamental change in the nucleic acids. In this species the macronucleus in the vegetative individual is a long and bent cylinder. During conjugation the macronucleus of both conjugants breaks up into a large number of fragments, and with the complete fusion of the conjugants to form a synconjugant (a phenomenon characteristic of all Peritricha) there are as many as 150 fragments of the old macronucleus filling the cyto-

some (3). Each is a spherical or nearly spherical solid body from 2 to 6 microns in diameter giving an intensely positive Feulgen reaction. As conjugation proceeds, and especially with the formation of the synkaryon and its divisions, each fragment develops a Feulgen negative space in its center, which at first small, becomes progressively larger. Towards the end of the conjugation process and with the reorganization fissions that follow it, the fragments are detectable only as very faintly staining ringlike structures which are soon absorbed in the cytoplasm (Figs. 1 to 4).

An understanding of this vacuolation of the macronuclear fragments was possible as a result of application of specific stains and digestion by nucleases. From what has been said above, the development of a Feulgen negative space in the center of each fragment would mean that the nucleic acid in this region is no longer of the desoxyribose type and progressively the DNA content of each fragment is being reduced until it is, in the final stages, represented by a very thin peripheral region. This is an interesting change, and to our knowledge, is being reported for the first time.

The determination of the precise nature of the change involved in this vacuolation was made possible by the application of Unna's pyronin-methyl green staining mixture. This showed the space in the center of each fragment filled with material which gave a reaction with pyronin while the peripheral region was stained with methyl green. With the progressive increase of the central pyronin stained region, the peripheral methyl green stained portion became thinner and less prominent. It is now widely recognized (2, 5) that RNA has a marked affinity for pyronin and it seemed to indicate that in the center of each fragment DNA was progressively being replaced by RNA.

A further test was applied and conjugation stages were subjected to ribonuclease digestion. In such preparations the central region of each fragment failed to stain with pyronin.

This seems to provide evidence that within each macronuclear fragment a conversion of DNA into RNA is taking place.

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A NOTE CONCERNING BACTERIAL CELL WALLS, MITOCHONDRIA AND NUCLEI

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In a recent article in this journal by Bisset (1) the following paragraphs appear:

"A somewhat different appearance is seen in Fig. 9, which represents the condition seen frequently in *M. tuberculosis*, when it is heat-fixed and stained. The cell contents have shrunken away from the unstained cell wall, and appear as a string of granules. In a recent study, Mudd *et al.* (11)[2] have chosen to interpret these as 'mitochondria'."

— — —

"If bacteria of this type are stained very briefly with aqueous basic dyes, the most obvious structures which appear are the growing points and cytoplasmic septa (Fig. 14). These have been confused with the nuclear structures by some observers (7), and with mitochondria (11).[2]"

— — —

"The misinterpretation of appearances of this type is, in the opinion of the author, responsible for reports of classical mitotic spindles in bacilli, extra cytoplasmic spore nuclei, mitochondria and other observations which are discussed in the text."

The structures in question are shown in electron micrographs in Figs. 1 to 5, so that the reader may form his own judgment concerning their reality and the validity of Bisset's assertions. The preparations shown, like those for electron micrographs published earlier (7, 8, 11), were neither heat-fixed nor stained.

Figs. 1 to 3 show cells of *Mycobacterium thamnopheos*. The dark, electron scattering granules are clearly distinct entities within the protoplasts; the protoplasts themselves are bounded by cytoplasmic membranes and by external cell walls. The protoplasts are shrunken by desiccation slightly away from the very delicate cell walls. In Fig. 3 accumulation of extrinsic material against the cell walls makes these walls particularly conspicuous. Nuclei are not clearly differentiated from the cytoplasm in such electron micrographs of mycobacteria, because of insufficient density-contrast.

Figs. 4 and 5 show a microcolony of virulent human tubercle bacilli growing in the serpentine cords characteristic of virulent *M. tuberculosis* (6). In Fig. 4 the bacilli have been subjected only to desiccation and fixation in osmic acid vapor.

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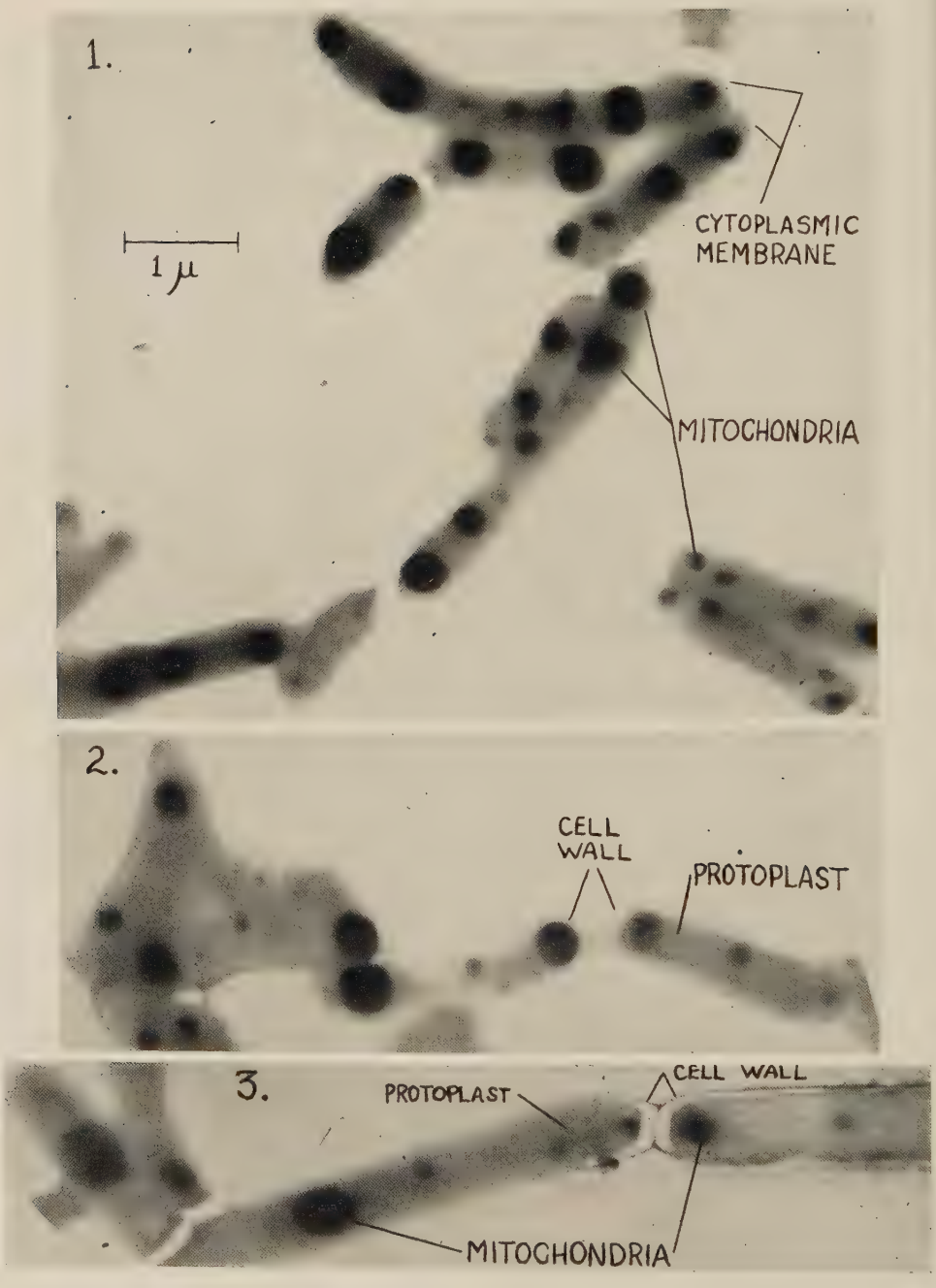
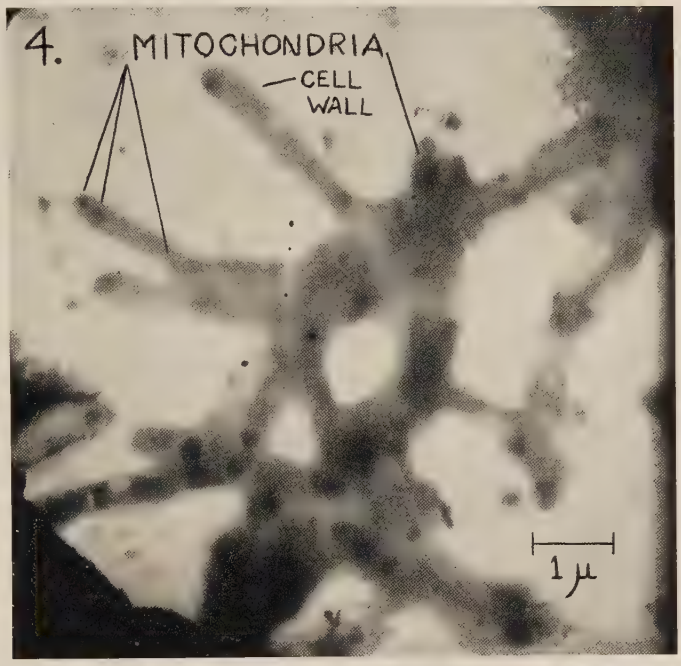
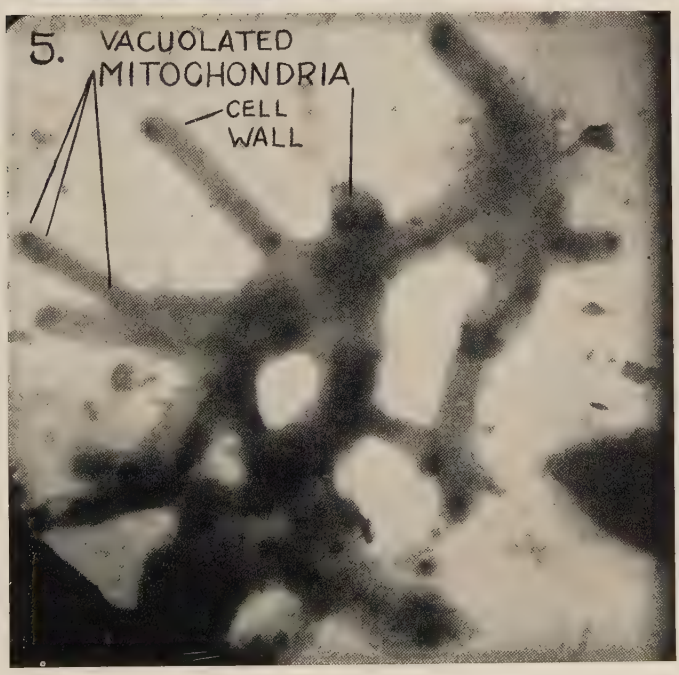
[1] Medical Research Fellow of the National Tuberculosis Association.

[2] Reference (11) in Bisset's article is reference (8) in this note.


1.
1 μ
CYTOPLASMIC MEMBRANE
MITOCHONDRIA
2.
CELL WALL
PROTOPLAST
3.
PROTOPLAST
CELL WALL
MITOCHONDRIA

Figs. 1–3 are of *M. thamnopheos* grown on collodion (3) overlying glycerol agar. Held in desiccator until electron micrographed. Magnifications as reproduced 15 000 ×.
Fig. 1. Grown on glycerol agar 6 hours.
Fig. 2. Grown 6 hours on glycerol agar containing 0.1 per cent triphenyltetrazolium chloride.
Fig. 3. Grown 24 hours on glycerol agar. Fixed for 1 minute in vapors over 2 per cent OsO_4 solution.





Figs. 4 and 5 of *M. tuberculosis hominis* from human case, proven virulent by guinea pig inoculation.
Fig. 4. Grown for 96 hours on collodion overlying glycerol agar. Held in desiccator until micrographed. Fixed 2 minutes in vapor over 2 per cent OsO_4 solution. Magnification as reproduced 11 000 ×.
Fig. 5. Same field as in Fig. 4 after intense electron bombardment.

The electron micrographs were taken by Mrs. Jean Minkin at the Laboratories of Research and Development, Franklin Institute, Philadelphia, Pennsylvania.

In Fig. 5 the same field has been subjected to intense electron bombardment. This brings out an additional characteristic of the electron scattering cytoplasmic granules of mycobacteria, namely that they can be volatilized and vacuolated by electron bombardment (4, 5, 8, 9, 10). The cell walls are not so volatilized. The positive morphological and cytochemical evidence that the electron-scattering, enzymatically active, cytoplasmic granules are in fact mitochondria has been set forth in detail, and will not be repeated here. See also DeLamater (2).

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A DIAMOND KNIFE FOR ULTRATHIN SECTIONING

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With recent improvements in microtomy for ultrathin sectioning (2, 4) the problem of knife edge quality and sharpness has become of increasing importance. Although special steel and glass knives (3) are generally satisfactory, their edges can be used for a limited time only and hard specimens can not be cut. In an attempt to overcome these drawbacks knife edges of harder crystalline materials have been suggested (1). Diamonds seem particularly suitable for this purpose, since exceedingly sharp cutting edges of unsurpassed hardness can be prepared. In ruling engines for optical gratings diamond edges of extreme sharpness and durability are therefore routinely employed. However, diamond edges are also very fragile and especially sensitive to impacts and small blows. After initial unsuccessful attempts, diamond edges have now been prepared which are of adequate sharpness for cutting ultrathin sections, and maintain this degree of sharpness practically unaltered over long periods of constant use. The natural cleavage planes of certain industrial diamonds (Brazilian Boarts) which are built up of submicroscopic layers can already be used for sectioning, but these edges are as a rule too irregular. By cleavage of these diamonds at certain angles and polishing of the resulting edge with special equipment[1] cutting edges of extraordinary sharpness and regularity can be obtained. In view of the regular crystalline structure of diamond it should eventually be possible to obtain edges of truly molecular thickness. The only limiting factor at present seems to be the grain of the polishing powders, and this can perhaps be overcome by electrical polishing. The diamonds (0.3 carat, 2.5 mm cutting edge; supplied by Industridiamanter A.B.) are set in a suitable alloy and mounted in a special knife holder (Fig. 1) which must be of great stability to avoid detrimental vibrations. After adequate polishing of the edge and determining the optimal cutting angle, serial ultrathin sections (0.5—0.1 μ) of fresh frozen tissues and of methacrylate embedded material have been cut with this knife (Fig. 2). Although still showing numerous knife tracks, these sections are comparable to the best sections obtained in this laboratory with steel and glass knives. The reproducibility of section thickness is satisfactory, and the chemically inert diamond edge is particularly suitable for cutting sections of fresh tissues without introducing contamination. The same diamond knife edge has been in constant use for several weeks without needing resharpening.

[1] The valuable advice of Professor Manne Siegbahn and the expert assistance of Mr. A. Trommer in polishing the diamond edges are gratefully acknowledged.

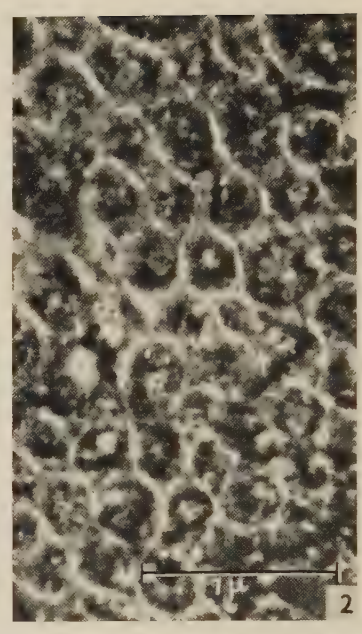

Fig. 1. Diamond knife mounted in special holder of the microtome used for cutting ultrathin frozen sections.
Fig. 2. Transverse section through a bundle of submicroscopic nerve fibres cut with the diamond knife. The thin sheaths of the nerve fibres appear as rings containing the sectioned axon filaments. Magnification: 25000 ×.

Preliminary trials indicate that ultrathin (fragmented) sections of hard materials like bone and certain metals can also be cut with the diamond knife. Improved diamond knives of this type appear to be ideal for ultrathin sectioning and for microtomy in general, since they combine extreme sharpness with utmost hardness and durability. Moreover, diamonds are inherently capable of giving sharper edges than any known material, once the polishing process has been perfected. Details of the process for preparing the diamond knives and the results obtained will be presented in a later publication.

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THE CHROMATIC NUCLEAR MEMBRANE

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In fixed and stained cells, the nuclear membrane has three components: the perinuclear layer, the achromatic membrane and the chromatic membrane (1). Little attention has been paid to the chromatic component which has been generally considered to be an artefact. Yet it has been observed in ultraviolet and phase contrast micrographs of living cells by Ludford and Smiles (18, 19) who described the nuclear membrane as a "hyaline film with chromatin spread unevenly over its internal surface". Moreover, in fixed material, it is a common observation that the prophase chromosomes arise in close relation to the nuclear membrane and incorporate the chromatic component into their structure. A similar intimate relation between the prophase chromosomes and the nuclear membrane has been observed in living cells (16). These observations indicate that the chromatic nuclear membrane is a definite cell structure and this conclusion is supported by the study of ultracentrifuged cells.

Experimental: Small pieces of rat liver were ultracentrifuged for two hours at 150 000 × g. in the rotor of an air-driven Beams ultracentrifuge (3, 4). The pieces were then fixed in Zenker-formol, dehydrated, embedded in paraffin wax and sections cut parallel to the direction of sedimentation. The sections were stained with Ehrlich's haematoxylin. Adjacent sections treated with the Feulgen reagent showed that all the nuclear structures stained with haematoxylin contained desoxyribonucleic acid.

Observations: Fig. 1 shows an interphase nucleus in which the deeply stained chromatic nuclear membrane can be clearly seen. The chromatin network has been sedimented to the centrifugal pole of the nucleus. Fine chromatic strands connect this mass of chromatin to the nuclear membrane. These chromatin threads are more clearly shown in the interphase nucleus depicted in Fig. 2. Fig. 3 is a micrograph of a binucleate hepatic cell with both nuclei synchronously in the late prophase of mitosis. Some of the chromosomes have been sedimented to the centrifugal pole of the nucleus, but a number of chromosomes have remained attached to the nuclear membrane and have failed to be dislodged. In this cell, the chromatic nuclear membrane is no longer continuous but is made up of the parts of chromosomes applied to the inside of the achromatic membrane.

Similar results have been recorded by previous investigators but the significance of the observations were not emphasised. In the centrifuged liver cells depicted by Claude (12), the interphase nuclei show chromatic strands connecting the chromatic

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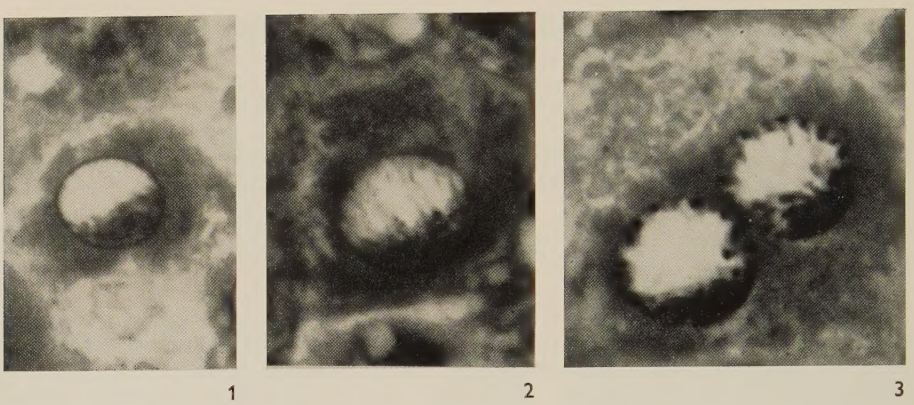

1 2 3

Figs. 1, 2 and 3. Photomicrographs of liver cells ultracentrifuged at 150 000 × g. for 2 hours. The direction of sedimentation is from the upper left to the lower right corner of each figure. Magnification × 2 000.

nuclear membranes with the sedimented chromatin. In addition, karyosomes have remained attached to the nuclear membrane undislodged by the centrifugal force. Claude (14) has recently recorded even clearer pictures showing these features. Beams (2) mentions that the displaced chromatin in the interphase nuclei of ultracentrifuged connective tissue cells is connected by threads to the nuclear nembrane.

The persistence of the chromatic nuclear membrane after the application of intense centrifugal force indicates that it is not an artefact. If it were merely a peripheral condensation of diffuse chromatin, then after ultracentrifugation it should surround the sedimented chromatin only. The fine chromatic threads which connect the centrifuged chromatin to the nuclear membrane lie parallel to the direction of sedimentation. This orientation precludes the possibility that they are fibres randomly produced by precipitation. Most probably these threads are the thin uncoiled chromosomes of the interphase nucleus. The failure of ultracentrifigation to dislodge the prophase chromosomes demonstrates that the relation between these chromosomes and the nuclear membrane is a particularly intimate one.

Discussion: All of these observations show that the chromatic nuclear membrane is a definite cell structure, composed of segments of chromosomes applied to the inside of the achromatic membrane. These segments maintain their continuity with the remaining parts of the chromosomes which, as uncoiled threads, occupy the inside of the interphase nucleus. Such an interpretation of the structure accounts for the observations that the nuclear membrane is formed during telophase at the peripheral interface between the chromosomes and the cytoplasm (22), and that the chromosomes appear in prophase in exactly the same position that they occupied during the previous telophase (5).

It should be pointed out that the recent electron micrograph studies of isolated nuclear membranes by Callan and Tomlin (9) and Bovey (8) showed no indication of a chromatic nuclear membrane. Only two layers were observed: an outer discontinuous layer corresponding to the perinuclear layer and an inner con-

tinuous achromatic membrane. However, the discrepancy between these results and the present observations is easily resolved. These investigators used cells which are in the prophase of meiosis (amphibian (9) and insect oocytes (8)) and the giant salivary nuclei of *Chironomus* (8); all of these cells contain fully organised chromosomes and therefore would not be expected to possess chromatic nuclear membranes. However, in sectioned *Amoeba proteus*, electron micrographs show a discontinuous layer on the *inside* of the continuous membrane (15) and it is possible that the former may represent the chromatic membrane.

The main conclusion to be drawn is that, during the interphase, there is an organised continuity of chromosomal segments on the nuclear membrane. Since this chromatic nuclear membrane retains its charge of desoxyribonucleic acid in interphase nuclei, it may be classified as heterochromatic. Heterochromatic regions of the chromosomes control the nucleic acid metabolism of the cell (10, 11, 23). Cytoplasmic nucleic acid has been shown to be concentrated perinucleally and synthesised in close relation to the nuclear membrane (10). According to Caspersson (10), the nucleolus and its associate heterochromatin play the decisive role in this process; from the nucleolus, diamino acid rich proteins diffuse out the nuclear membrane and induce there the synthesis of ribonucleic acid. However, it is reasonable to suggest as an alternative hypothesis that the synthesis of cytoplasmic ribonucleoprotein is under the direct control of the heterochromatin of the nuclear membrane, and that this process occurs in parallel with the synthesis of nucleolar ribonucleic acids by the nucleolar-organising heterochromatin within the nucleus. Cytoplasmic ribonucleic acid as visualised by cytochemical techniques in the hepatic cell is identical with the microsomes (7) in which the nucleic acid is complexed with lipoproteins and enzymes (13). The microsomes play a fundamental role in protein synthesis and the general metabolism of the cell (6).

The above hypothesis provides a basis for explaining the mediation of the specific effects of the nucleus in the cytoplasm. It is proposed that the nuclear membrane heterochromatin with its specific genes controls the synthesis of equally specific microsomes, each desoxyribonucleic acid directing the synthesis of a characteristic type of ribonucleoprotein. Variation of the nuclear membrane heterochromatin pattern from cell type to cell type would be associated with the origin of cell specificity during differentiation. Indeed, such a possible role for the nuclear membrane was proposed by Schultz (24), but he considered at the time that there was insufficient evidence for this possibility. Since the interphase chromosomes contain ribonucleic acid (20), it is probable that ribonucleoproteins are synthesised within the heterochromatic segments and then pass through the achromatic membrane into the cytoplasm as suggested by the studies of Jeener and Szafarz (17). On the other hand, the operative mechanism may be a trans-membrane synthesis of the type considered feasible by Rothen (21).

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